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LA PROTEINE HSP110 : ROLE DANS LE DEVELOPPEMENT TUMORAL ET SUR L'IMMUNOGENICITE DU CANCER COLORECTAL

Présentée et soutenue publiquement par

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Résumé

Notre équipe étudie les HSP, et notamment HSP110. Les HSP sont des chaperons impliqués dans le repliement des protéines nouvellement synthétisées et dénaturées. Les HSP sont surexprimées lors des stress et participent à la survie des cellules par leurs propriétés anti-apoptotiques et anti-agrégations. HSP110 est surexprimée dans le cancer colorectal et est associée à un mauvais pronostic. L'expression d'un mutant d'HSP110, nommé HSP110ΔE9, a été mise en évidence dans les cancers colorectaux de type MSI. Celui-ci y agit comme un dominant négatif, en se liant à HSP110 et en inhibant ses fonctions. Son expression sensibilise les cellules cancéreuses à la chimiothérapie et est associée à un bon pronostic chez les patients.

Je me suis tout d'abord intéressé au rôle d'HSP110 dans la régulation de la voie oncogénique STAT3. Son activation est en effet associée à un mauvais pronostic, par l'induction de gènes impliqués dans la prolifération et la survie. La protéine HSP110 favorise la prolifération des cellules colorectales cancéreuses à travers cette voie. HSP110ΔE9 en revanche l'inhibe.

Je me suis ensuite intéressé au rôle d'HSP110 sur la polarisation des macrophages dans le cancer colorectal. Celle-ci peut être sécrétée par les cellules cancéreuses et induit une polarisation pro-tumorale des macrophages. HSP110ΔE9, en bloquant la sécrétion d'HSP110, conduit en revanche à une polarisation pro-inflammatoire. L'effet d'HSP110 sur la polarisation implique le récepteur TLR4.

L'ensemble de ces résultats montrent le rôle d'HSP110 dans la progression tumorale. HSP110 apparaît comme une cible thérapeutique dans le traitement du cancer colorectal.

Abstract

Our team studies HSPs, including HSP110. HSPs are chaperones involved in the folding of newly synthesized and denatured proteins. HSPs are overexpressed under stress conditions and are involved in cell survival thanks to their anti-apoptotic and anti-aggregation functions. HSP110 is overexpressed in colorectal cancer and is associated with a poor prognosis. The expression of a mutant HSP110, named HSP110ΔE9, has been shown in MSI colorectal cancer. This one was shown to act there as a dominant negative, by binding HSP110 and inhibiting its functions. Its expression sensitizes cancer cells to chemotherapy and is associated with a better prognosis for patients.

I was first interested in HSP110 role in regulating the oncogenic STAT3 pathway. Its activation is associated with a poor prognosis, as it induces the transcription of genes involved in proliferation and survival. HSP110 favors colorectal cancer cell proliferation through this pathway. Conversely, HSP110ΔE9 inhibited it.

I then focused on the role of HSP110 on macrophage polarization in colorectal cancer. HSP110 can be secreted by cancer cells and induces a pro-tumoral macrophage polarization. In contrast, HSP110ΔE9, by inhibiting HSP110 release, leads to a pro-inflammatory polarization. HSP110 effect on macrophage polarization involve the TLR4 receptor.

All these results show HSP110 role in tumor progression. HSP110 appear as a therapeutic target in the treatment of colorectal cancer.

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Liste des abréviations

17-AAG: 17-Allylamino-17-demethoxygeldanamycine
17-DMAG: 17-Dimethylamino-ethylamino-17demethoxygeldanamycine
5-FU: 5-Fluorouracile

ACTRII: Activin receptor II
ADCC: Antibody-dependent cell-mediated cytotoxicity
ADN: Acide désoxyribonucléique
ADP: Adénosine diphosphate
APAF1: Apoptotic peptidase activating factor 1
APC: Adenomatous polyposis coli
APE1/Ref-1: Apurinic apyrimidinic endonuclease redox effector factor-1
APG: ATP and peptide binding protein in germ cells
ARNm: Acide ribonucléique messager
ATP: Adenosine triphosphate

BAG1: BAG family molecular chaperone regulator 1
BAX: Bcl-2–associated X protein
Bcl: B-cell lymphoma
BLM: Bloom syndrome protein

CACNA1G: Calcium channel, voltage-dependent, T type, alpha 1G subunit
CBP: CREB-binding Protein
CCL: Chemokine ligand
CCR: Chemokine receptor
Cdk: Cyclin-Dependent Kinase
CEA: Carcinoembryonic antigen
CIMP: CpG island methylator phenotype
CIN: Chromosomal instability
CIS: Cytokine-inducible SH2 proteins
CMH: Major histocompatibility complex
cMoP: Common monocyte progenitor
CMP: Common myeloid progenitor
CNTF: Ciliary neurotrophic factor
CPA: Cellules présentatrices d'antigènes
CRC: Cancer colorectal
CSF-1: Colony stimulating factor 1
CSF-1R: Colony stimulating factor 1 receptor
CTLA-4: Cytotoxic T lymphocyte–associated antigen
CX3CL1: Chemokine (C-X3-C motif) ligand 1
CX3CR1: CX3C chemokine receptor 1

EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EMAST: Tetranucleotide repeats
EXO1: Exonuclease 1

Fas-L: Fas ligand
FcγRIII: Fc receptor
FGFR: Fibroblast growth factor receptor
FOXP3: Forkhead box P3

GM-CSF: Granulocyte-macrophage colony-stimulating factor
GMP: Granulocyte-Macrophage progenitor
Gp: Glycosylated protein
GRP: Glucose-related protein

Hck: Hematopoietic cell kinase
HER-2/neu: Human Epidermal Growth Factor Receptor 2
HIF: Hypoxia inducible factor
HNPCC: Hereditary non polyposis colorectal cancer
HSC: Hematopoietic stem cell
HSC70: Heat shock cognate 70 kDa protein

HSE: Heat shock element
HSF: Heat shock factor
HSP: Heat shock protein
HspBP1: Hsp70-binding protein 1

IDO: Indoleamine 2, 3-dioxygenase
IFN: Interferon
IGF2: insulin-like growth factor
IGFIIR: insulin-like growth factor receptor
IL: Interleukin
IRF: Interferon regulatory factor
ISRE: Interferon-stimulated response element

JAK: Janus tyrosine kinase

KIR: Killer inhibitory receptor

Lck: Lymphocyte-specific protein tyrosine kinase
LEF: Lymphoid enhancer-binding factor 1
LIF: Leukemia inhibitory factor
LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1
LPS: lipopolysaccharide

MafB: V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK: Mitogen-activated protein kinase
MBD4: Methyl-CpG-binding domain protein 4
Mcl-1: Myeloid cell leukemia 1
MDP: Macrophage and DC common precursor
MDSC: Monocyte-Derived Suppressor Cell
MLH: MutL homolog
MMP: Matrix metalloproteinases
MMR: Mismatch repair
MSH: MutS homolog
MSI: Microsatellite instability
MSS: Microsatellite stability
mTOR: Mammalian target of rapamycin

MUC-1: Mucin-1

NBD: Nucleotide binding domain
NcoA: Nuclear receptor coactivator 1
NEF: Nucleotide exchange factor
NES: Nuclear export sequence
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NK: Natural killer
NLS: Nuclear localization sequence
NMD: Nonsense mediated decay
NO: Nitric oxide

OGT: O-GlcNAc transferase
OSM: Oncostatin M

PAF: Familial adenomatous polyposis
PAMP: Pathogen-associated molecular pattern
PD-1: Programmed death-1
PDGF: Platelet-derived growth factor
PDGFR: Platelet-derived growth factor receptor
PD-L1: Programmed death-ligand 1
PES: 2-Phenylethynesulfonamide
PIAS: Protein inhibitors of activated STATs
PKC: Protein kinase C
PP2A: Protein phosphatase 2
PTEN: Phosphatase and tensin homolog
PTP: Protein tyrosine phosphatase

ROS: Reactive oxygen species

SH2: Src-homology 2
SHP: Protein tyrosine phosphatase
SOCS: Suppressors of cytokine signaling
SR-A: Scavenger receptor A
SREC-1: Scavenger receptor expressed by endothelial cells 1
STAT: Signal transducer and activator of transcription

TAP2: Antigen peptide transporter 2

TC45: T-cell protein tyrosine phosphatase
TCF: T-cell factor
TGF: Transforming growth factor
TGFβRII: Transforming growth factor, beta receptor II
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor α
TPR: Tetratricopeptide repeat
TRAF6: TNF receptor-associated factor 6
TRAIL: TNF-related apoptosis-inducing ligand

VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor

WISP-3: WNT1-inducible-signaling pathway protein 3

Introduction

Chapitre I. La protéine HSP110

1. Les protéines de choc thermique

1.1. La réponse cellulaire au stress

Le maintien de l'homéostasie cellulaire est essentiel pour le bon fonctionnement des cellules. Celles-ci sont cependant continuellement soumises à différentes agressions qui menacent leur intégrité. L'état de stress des cellules peut être provoqué par de nombreux facteurs environnementaux (choc thermique, choc oxydatif, ultraviolet), l'exposition à des agents pharmacologiques (métaux lourds, alcool, agents oxydants, chimiothérapies anticancéreuses), ou encore par certaines conditions pathologiques (fièvre, inflammation). Ces stress physiques, chimiques ou physiologiques entraînent une modification de l'expression de certains gènes, ce phénomène est appelé « réponse au choc thermique » ou « réponse au stress ».

La réponse au choc thermique a été mise en évidence en 1962 par Ferrucio Ritossa suite à l'observation d'un renflement des chromosomes géants des glandes salivaires de drosophile après l'augmentation accidentelle de la température de son incubateur (Ritossa, 1996). L'activation chromosomique a ensuite été corrélée à l'expression de protéines de thermorésistance appelées « protéines de choc thermique » ou HSP (Tissieres et al., 1974).

Les HSP sont des protéines cellulaires très conservées d'une espèce à l'autre et sont retrouvées aussi bien chez les bactéries, les levures, les plantes, les animaux que chez l'homme. Alors que les HSP représentent déjà 2 à 3% des protéines cellulaires totales à l'état basal, leur expression est fortement induite en condition de stress. L'induction des gènes des HSP nécessite l'activation et la translocation au noyau de facteurs de transcription spécifiques, les « heat Shock Factors » (HSF). Les HSF se lient à l'ADN sur des séquences particulières, les « Heat Shock Element » (HSE), présentes en plusieurs copies dans le promoteur des gènes des HSP permettant ainsi leur expression (Akerfelt et al., 2010).

Les HSP sont retrouvées dans tous les compartiments cellulaires (cytoplasme, noyau, mitochondrie, réticulum endoplasmique, lysosomes…) et agissent comme des chaperons moléculaires (Figure 1). Afin de maintenir l'homéostasie cellulaire, les HSP prennent en charge la conformation tridimensionnelle des protéines nouvellement formées ou anormales et

participent à la formation de complexes multi-protéiques en condition physiologique ou suite à un stress (Beckmann et al., 1990; Gething and Sambrook, 1992). Les HSP peuvent participer au transport de leur substrat au travers des compartiments cellulaires, ainsi que dans la régulation de voies de signalisation (Brodsky et al., 1993; Haas, 1995). Si les HSP échouent à replier et maintenir la bonne conformation de protéines anormales, elles peuvent alors les orienter vers la voie de dégradation du système ubiquitine/protéasome (Lanneau et al., 2010). Lors d'un stress engageant la mort cellulaire programmée, l'apoptose, les HSP peuvent bloquer les voies de signalisation apoptotiques à différents niveaux et favoriser les voies de signalisation de survie cellulaire (Lanneau et al., 2008). Les HSP interviennent finalement dans la différenciation et l'érythropoïèse en régulant la stabilité ou la localisation de protéines et facteurs de transcription clefs (Didelot et al., 2008; Jego et al., 2014; Ribeil et al., 2007).

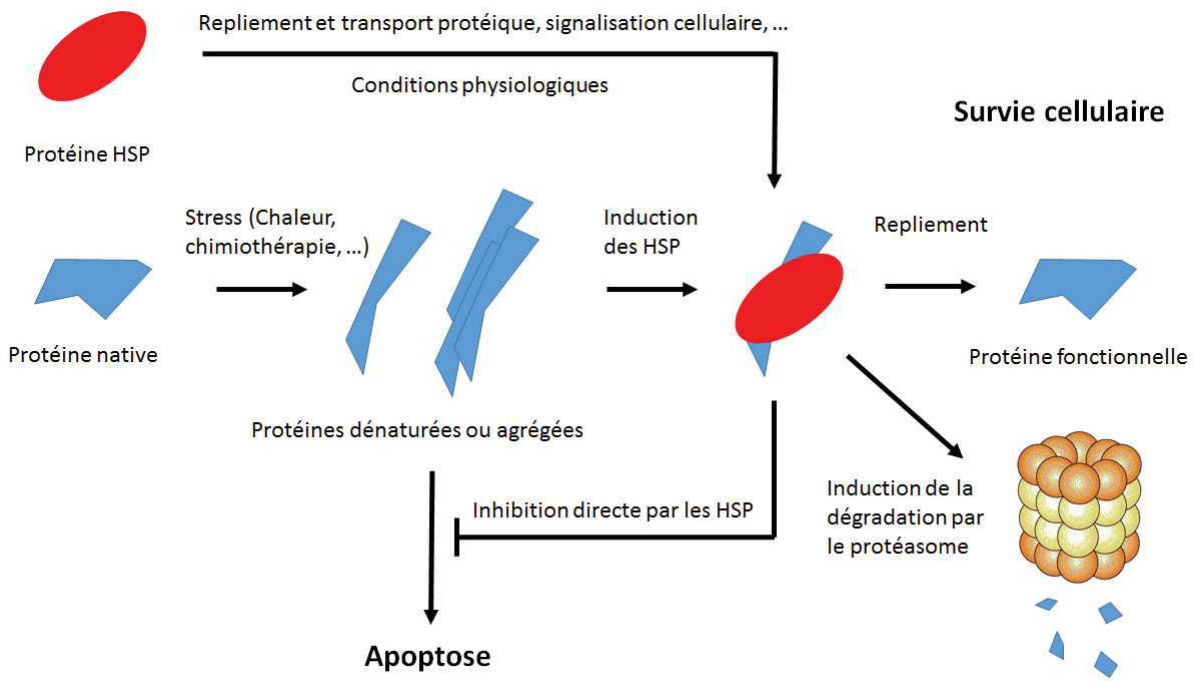

Figure 1 : Rôle des HSP en condition normale et en condition de stress.
En condition normale, les HSP participent au repliement des protéines nouvellement formées et à la signalisation cellulaire. En condition de stress, les HSP sont induites, interagissent avec les protéines dénaturées et entraînent leur repliement ou leur dégradation, permettant la survie cellulaire. Si le stress est trop important, les signaux apoptotiques prennent le dessus sur les signaux liés à la survie, conduisant à l'apoptose de la cellule.

1.2. Classification des HSP

Chez les mammifères, les HSP sont principalement réparties en 6 familles en fonction de leur poids moléculaire. On retrouve ainsi les familles HSP110, HSP90, HSP70, HSP60, HSP40 et la famille des petites HSP, comprenant notamment HSP27.

Les HSP ont d'abord été identifiées par leur poids moléculaire. A cause de la multiplication des membres d'une même famille au cours de l'évolution, cette nomenclature a donné lieu à de nombreuses confusions. Une même protéine peut être désignée de façon différente en fonction des espèces (Ex : HSP25 chez la souris et HSP27 chez l'homme et le rat) ou selon les auteurs en fonction de la variation dans la détermination des masses moléculaires (Ex : HSP70 et HSP72). Une même HSP peut avoir jusqu'à 10 noms différents (Ex : HSP90α). Ainsi, depuis quelques années, une nomenclature internationale pour les HSP tente d'être établie par le « *HUGO Gene Nomenclature Committee* » (Kampinga et al., 2009). Cette nouvelle nomenclature ne fait pas l'unanimité quant à l'utilisation d'une seule dénomination par HSP mais permet en revanche, lorsque l'on rencontre la dénomination d'une HSP, de connaitre tous ses autres noms (Figure 2).

Bien que les HSP partagent un grand nombre de propriétés communes, chaque famille présente des particularités qui leur sont propres telles que leur localisation cellulaire, leur expression (constitutive ou fortement inductible), leur dépendance ou non à l'ATP, leur spécificité de substrat ou encore le type de pathologies dans lesquelles elles peuvent être impliquées.

Gène	Protéine	Anciens noms	ID du gène humain
La famille des petites protéines de stress : HSPB			
HSP1	HSPB1	HSP27, HSP25	3315
HSP2	HSPB2	MKBP, HSP27	3316
HSP3	HSPB3	HSPL27	8988
HSP4	HSPB4	α-A cristallin, CRYAA, CRYA1	1409
HSP5	HSPB5	α-B cristallin, CRYAB, CRYA2	1410
HSP6	HSPB6	HSP20	126393
HSP7	HSPB7	cvHSP	27129
HSP8	HSPB8	H11, HMN2, HSP22	26353
HSP9	HSPB9	LJ27437	94086
HSP10	HSPB10	ODF, ODF1, RT7, ODF2, SODF	4956
HSP11	HSPB11	HSP16.2	51668
La famille HSP70 : HSPA			
HSPA1A	HSPA1A	HSP70, HSP70-1, HSP72	3303
HSPA1B	HSPA1B	HSP70-2	3304
HSPA1L	HSPA1L	HSC70t, hum70t, HSP-hom	3305
HSPA2	HSPA2	HSP70.2	3306
HSPA5	HSPA5	BIP, Grp78, MIF2	3309
HSPA6	HSPA6	Heat-shock protein-6	3310
HSPA7	HSPA7	Heat-shock protein-7	3311
HSPA8	HSPA8	HSC70, HSP71, HSP73	3312
HSPA9	HSPA9	Grp75, Mortalin	3313
HSPA12A	HSPA12A	FLJ13874	259217
HSPA12B	HSPA12B	RP23-32L15.1	116835
HSPA13	HSPA13	Stch	6782
HSPA14	HSPA14	HSP70-4, HSP70L1	51182
La famille HSP90 : HSPC			
HSPC1	HSPC1	HSP90α, HSP86, HSP90AA1, HSP90A	3320
HSPC2	HSPC2	HSP90AA2	3324
HSPC3	HSPC3	HSP90β, HSP84, HSP90AB1, HSP90B	3326
HSPC4	HSPC4	Gp96, grp94, TRA1, endoplasmin	7184
HSPC5	HSPC5	TRAP1, HSP75, HSP90L	10131
La famille HSP110 : HSPH			
HSPH1	HSPH1	**HSP105, HSP110**	10808
HSPH2	HSPH2	HSPA4, APG-2	3308
HSPH3	HSPH3	HSPA4L, APG-1	22824
HSPH4	HSPH4	HYOU1, Grp170, ORP150	10525

Figure 2 : Nomenclature des principales familles d'HSP (sHSP, HSP70, HSP90 et HSP110). (adapté de Kampinga et al., 2009).

1.3. Les HSP comme cibles thérapeutiques dans le traitement du cancer

De nombreux cancers, tels que les mélanomes, les cancers colorectaux, de la prostate, du sein et du poumon, sont caractérisés par une surexpression des HSP (Bauer et al., 2012; Huang et al., 2005; Katsogiannou et al., 2015; Lebeau et al., 1991; Protti et al., 1994). Cette surexpression est généralement associée à un mauvais pronostic pour les patients (Storm et al., 1996). Les cellules tumorales sont en effet dépendantes de ces protéines pour leur survie et leur développement, ces cellules étant continuellement stressées en raison de différents facteurs (accumulation de protéines mutées, forte prolifération, hypoxie,…).

Les mécanismes favorisant la surexpression des HSP dans le cancer sont globalement mal connus et concernent principalement le facteur de transcription HSF1 (Figure 3).

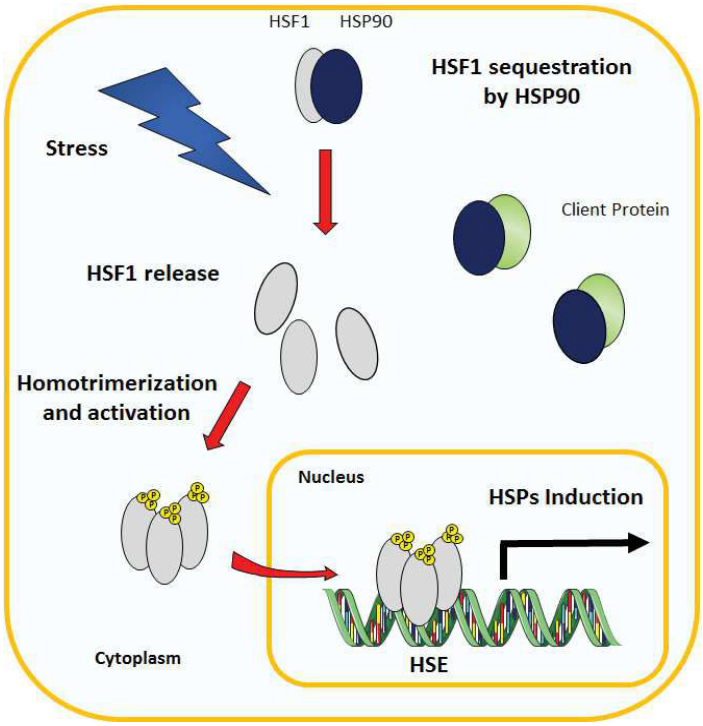

Figure 3 : Induction par HSF1 de l'expression des HSP.
Le facteur de transcription HSF1 est séquestré par la protéine HSP90 en condition normale. Au cours d'un stress, celui-ci se retrouve libre et s'homotrimérise. Il est alors activé par phosphorylation par différentes voies de signalisation. Il entre dans le noyau et induite la transcription des gènes des HSP.

Le facteur de transcription HSF1 est en effet suractivé dans les cellules cancéreuses, en raison de son hyperphosphorylation par l'activation de différentes voies de signalisation tumorigéniques (Khaleque et al., 2005; Murshid et al., 2010; Zhang et al., 2011). HSF1 peut également être surexprimé dans les cellules cancéreuses (Santagata et al., 2011). Cette surexpression pourrait être due à des dérégulations épigénétiques. En effet, le gène *HSF1*

contient plusieurs ilots CpG qui pourraient contribuer à la régulation de son expression en condition normale (Singh et al., 2009). La déméthylation de ces séquences durant la progression tumorale pourrait entrainer sa surexpression (Jones and Baylin, 2002).

1.3.1. Fonctions des HSP dans le cancer

Les HSP peuvent intervenir de différentes façons à travers leurs multiples fonctions dans la tumorigenèse.

Les HSP participent tout d'abord à la croissance tumorale par la stabilisation de protéines oncogéniques. HSP90 peut ainsi stabiliser de nombreuses protéines clientes impliquées dans la prolifération cellulaire telles que c-Src, STAT3, Raf-1 et HER2/neu (Blagosklonny, 2002). Ces protéines peuvent de plus séquestrer différents intermédiaires de la voie de l'apoptose afin de l'inhiber, augmentant ainsi la résistance des cellules cancéreuses à la chimiothérapie (Garrido et al., 2006).

Les HSP peuvent également promouvoir l'angiogenèse. La capacité des cellules cancéreuses à proliférer et à résister à l'apoptose n'est pas suffisante pour leur développement. Celles-ci nécessitent également la formation de nouveaux vaisseaux sanguins afin d'être approvisionnées en oxygène et en nutriments. HSP70 et HSP90 peuvent ainsi chaperonner le facteur de transcription HIF-1α (Neckers and Ivy, 2003). Le facteur de transcription HIF-1α est impliqué dans le contrôle de l'homéostasie de l'oxygène et est nécessaire pour la production de VEGF, un puissant agent angiogénique dans le cancer en condition d'hypoxie (Semenza, 2002). HSP90 est également impliquée dans la synthèse du VEGF (Sun and Liao, 2004). La sécrétion d'HSP27 par les cellules cancéreuses joue finalement un rôle clef dans la production de VEGF par les cellules endothéliales (Thuringer et al., 2013).

Les HSP sont impliquées dans la formation des métastases. Une corrélation entre l'expression des HSP27 et HSP70 avec le pouvoir métastatique est ainsi observée dans les études cliniques (Bausero et al., 2006; Gibert et al., 2012; Gong et al., 2015). Les cellules tumorales peuvent sécréter des protéines protéolytiques telles que les métalloprotéinases (MMP) afin de dégrader et remodeler la matrice extracellulaire, contribuant ainsi aux capacités invasives et métastatiques des tumeurs. HSP27 est ainsi capable d'activer la MMP2, et est impliquée dans la régulation de l'expression de protéines d'adhésion à travers son interaction avec la β-caténine (Fanelli et al., 2008; Xu et al., 2006).

Les HSP jouent finalement un rôle complexe dans l'immunité tumorale. En effet, les complexes HSP-antigènes extraits de tumeurs peuvent être employés comme des vaccins anti-cancéreux et entraîner une régression tumorale (Murshid et al., 2011). Les HSP sécrétées par les cellules tumorales peuvent en revanche avoir un rôle immunosuppresseur. La sécrétion d'HSP70 par les cellules cancéreuses colorectales peut ainsi activer les cellules myéloïdes suppressives (MDSC) et inhiber l'activation des lymphocytes T (Chalmin et al., 2010; Mambula and Calderwood, 2006). La sécrétion d'HSP27 peut, quant à elle, entrainer une polarisation pro-tumorale des macrophages (Banerjee et al., 2011).

1.3.2. Développement d'inhibiteurs des HSP

L'implication des HSP dans ces multiples processus tumorigéniques a conduit au développement d'inhibiteurs de ces protéines. Des dérivés de la geldanamycine, une benzoquinone ayant des propriétés anti-tumorales mais présentant une forte toxicité, ont par exemple été développés. Ces molécules ciblent le domaine de liaison à l'ATP d'HSP90 avec une meilleure affinité que les nucléotides naturels, empêchant ainsi ses changements conformationnels et son activité chaperon. L'un de ces dérivés, le 17-AAG, est ainsi capable de réduire la croissance tumorale, la formation de métastases, et sensibilise les cellules à la chimiothérapie (Roh et al., 2013; Ye et al., 2015). Cet inhibiteur est actuellement en phase II (Gartner et al., 2012; Solit et al., 2008). Le 17-DMAG, quant à lui, montre également des effets anti-tumoraux dans différentes types de tumeurs solides et leucémiques et présente en plus une meilleure solubilité que le 17-AAG. Celui-ci est actuellement en essai clinique de phase I (Kummar et al., 2010; Lancet et al., 2010).

Des molécules chimiques pouvant cibler et inhiber les différents domaines d'HSP70 ont également été développées. L'interaction du PES avec le domaine chaperon d'HSP70 entraîne l'agrégation des protéines puis la mort cellulaire (Steele et al., 2009). De la même manière que pour les inhibiteurs d'HSP90, certaines molécules chimiques, telles que le VER-155008, ciblent son domaine de liaison à l'ATP et empêchent la protéine de réaliser son cycle (Massey et al., 2010). Notre équipe a récemment développé des aptamères peptidiques capables de cibler ce même domaine. Ces aptamères sensibilisent les cellules à la chimiothérapie et ont un fort effet anti-tumoral *in vivo* (Rerole et al., 2011).

Des inhibiteurs d'HSP27 sont également en développement. L'OGX-427, un oligonucléotide anti-sens ciblant HSP27, est actuellement en essai clinique de phase II (Rexer, 2011). Son utilisation dans les cancers pancréatiques, du poumon et de la prostate limite la croissance tumorale, la formation de métastases et sensibilise les cellules tumorales aux agents chimiothérapeutiques (Baylot et al., 2011; Lelj-Garolla et al., 2015; Rocchi et al., 2006).

Des effets de compensation peuvent cependant se produire dans les cellules cancéreuses suite à l'inhibition d'une HSP. L'inhibition d'HSP90 entraîne, par exemple, une surexpression d'HSP70, limitant ainsi l'effet du traitement. L'utilisation de combinaison d'inhibiteurs d'HSP est donc une stratégie envisageable. (Kuballa et al., 2015).

Alors que les protéines des familles HSP90, HSP70 et des petites HSP sont les plus connues, les protéines de la famille HSP110 ont été peu étudiées et considérées comme ayant des fonctions proches de celles d'HSP70, en raison de leurs homologies de structure, jusqu'au début des années 1990. Il est maintenant établi que les protéines de cette famille présentent des fonctions distinctes. (Easton et al., 2000). HSP110 est retrouvée surexprimée dans différents cancers mais son implication dans la progression tumorale est à ce jour peu connue.

2. Les caractéristiques d'HSP110

2.1. Expression

La protéine HSP110 appartient à la famille des HSP de très haut poids moléculaire, comprenant 4 membres chez les mammifères, HSP110, APG-1, APG-2 et GRP170.

HSP110, aussi nommée HSP105, est la 3ème HSP la plus abondante dans de nombreux types cellulaires et tissus, après HSP70 et HSP90 (Landry et al., 1982; Levinson et al., 1980; Subjeck et al., 1982a; Subjeck et al., 1982b; Tomasovic et al., 1983). Elle peut représenter jusqu'à 0,7% des protéines totales après choc thermique (Subjeck et al., 1982b). Deux formes d'HSP110, issues d'un épissage alternatif, ont été identifiées et nommées HSP105α et HSP105β, cette dernière étant plus petite de 43 acides aminés. HSP105α est localisée à la fois dans le noyau et le cytoplasme. HSP105β est, en revanche, majoritairement localisée dans le

noyau et uniquement retrouvée en condition de choc thermique. Les différences de fonctions entre ces deux protéines ne sont pas connues (Yasuda et al., 1995).

L'expression constitutive d'HSP110 diffère selon les organes. Son expression est faible dans les muscles squelettiques et le cœur mais forte dans le foie, les gonades et le cerveau (Lee-Yoon et al., 1995; Yasuda et al., 1995). Le cervelet des mammifères exprime cependant faiblement HSP110 (Hylander et al., 2000). De façon intéressante, le cervelet est particulièrement sensible à l'hyperthermie et aux effets toxiques associés à l'alcool (Albukrek et al., 1997; Manto, 1996). L'absence d'HSP110 pourrait contribuer à cette sensibilité.

L'expression d'HSP110 peut être induite par différents stress tels que la chaleur, l'éthanol, les agents oxydants et l'inflammation. L'analyse de la séquence génomique murine d'HSP110 a permis d'identifier sur son promoteur deux séquences HSE, permettant la liaison d'HSF1 en réponse au choc thermique (Yasuda et al., 1999). L'utilisation de souris déficientes en HSF1 montre que celui-ci est essentiel pour l'induction d'HSP110 par le choc thermique, tout comme pour celle d'HSP70 et d'HSP27 (Zhang et al., 2002). La présence d'une séquence de fixation du facteur de transcription STAT3 a également été mise en évidence dans le promoteur du gène humain (Olszak et al., 2014). L'expression d'HSP110 peut être induite par l'oncogène E7 du papillomavirus humain, un facteur de transcription viral. Cette induction nécessite la présence de la région conservée 2 de E7, qui est essentielle à la liaison de celui-ci aux protéines de la famille du rétinoblastome. L'induction d'HSP110 pourrait donc être coordonnée avec l'initiation du cycle cellulaire et impliquée dans la transformation de la cellule par le virus (Morozov et al., 1995).

Les protéines APG-1 et APG-2 ont été identifiées chez l'homme et la souris. Ces deux protéines sont principalement exprimées dans les gonades, mais également dans les autres tissus à un faible niveau. APG-1 est surexprimé au cours de la maturation des cellules germinales, suggérant un rôle dans leur développement. APG-1 est inductible par la chaleur dans les cellules somatiques (et non germinales). La condition optimale d'induction de son expression nécessite le passage de la température de 32 à 39°, contrairement au passage de 37 à 42° pour les autres HSP. APG-2 n'est, quant à elle, pas inductible par la chaleur (Kaneko et al., 1997a; Kaneko et al., 1997b; Kaneko et al., 1997c).

La protéine Grp170 est, quant à elle, localisée essentiellement dans le réticulum endoplasmique. Son expression peut être induite par différents stress tels que la privation de glucose, un pH acide ou l'anoxie (Lin et al., 1993).

2.2. Structures secondaires d'HSP110

Les protéines de la famille d'HSP70 ont été bien étudiées et ont été modélisées chez les eucaryotes et les bactéries. Des études de diffraction aux rayons X de la DnaK, l'homologue d'HSP70 chez la bactérie, ont permis d'établir sa structure tridimensionnelle (Zhu et al., 1996). La similarité des séquences d'HSP110 et de la DnaK a permis d'aligner les structures secondaires prédites d'HSP110 aux structures secondaires de la modélisation cristallographique de la DnaK afin d'établir sa structure tridimensionnelle (Oh et al., 1999).

HSP110 est ainsi composée de 4 domaines (Figure 4) :

- Un domaine de liaison à l'ATP (résidus 1-394)
- Un domaine de liaison au peptide (résidus 394-509), composé de 7 brins β majeurs formant un β-sandwich. La sérine en position 509 peut être phosphorylée par la caséine kinase 2 (Ishihara et al., 2003). Le rôle de cette phosphorylation n'est cependant pas connu. On la retrouve surtout dans le cerveau chez la souris. Elle pourrait jouer un rôle important dans cet organe (Ishihara et al., 2000).
- Un domaine Loop (résidus 510-608), composé de plusieurs acides aminés chargés négativement. Sa structure et sa fonction ne sont pas connues. Ce domaine comporte un signal de localisation nucléaire (NLS) situé entre les acides aminés 583 et 590.
- Un domaine C-terminal (résidus 608-858), composé d'une série d'hélices α et comportant un signal d'export nucléaire (NES) localisé entre les acides aminés 607 et 617. Les membres de la famille HSP110 montrent un haut degré d'homologie de séquence dans cette région (Lee-Yoon et al., 1995). Toutes les protéines de cette famille possèdent dans ce domaine un motif (DLD, DVD,…), très similaire au motif EEVD d'HSP70. Ce dernier est responsable de l'interaction d'HSP70 avec des protéines contenant des domaines répétés tetratricopeptide (TPR). HSP110, tout comme HSP70, possède également deux motifs consensus conservés dans l'hélice B et C. Le premier motif, nommé « Magic » (LEKERNDAKNAVEECVY), est situé entre les acides aminés 626 et 644. Le second, nommé « TedWlyee » (TEDWLYEEGEDQAKQAY), est localisé entre les acides aminés 673 et 689 (Easton et al., 2000). Ces motifs sont impliqués dans la reconnaissance et la liaison de substrats ou de cochaperons. Le domaine C–terminal de la DnaK forme un couvercle au-dessus du domaine de liaison du peptide, régulant l'entrée et/ou la sortie du substrat (Zhu et al., 1996). Ce couvercle

est particulièrement étendu chez les protéines de la famille d'HSP110, comparativement à celui des protéines de la famille d'HSP70.

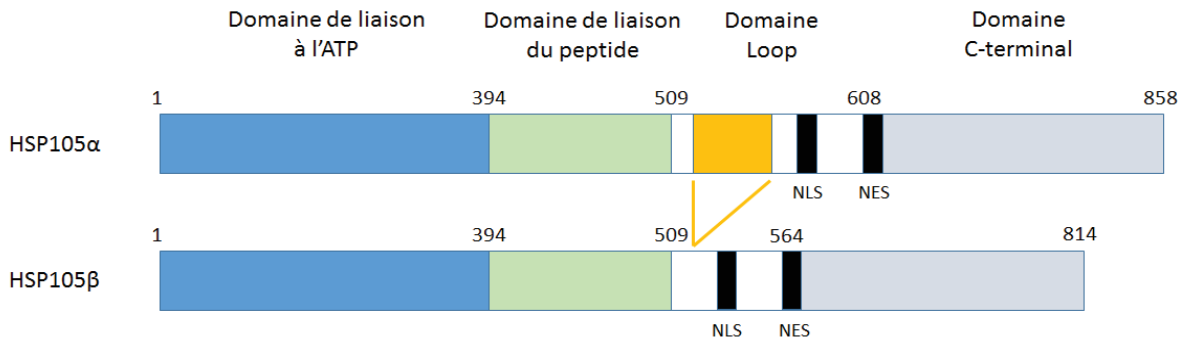

Figure 4 : Structure de la protéine HSP110.
La protéine HSP110 est une protéine composée de 4 domaines : un domaine de liaison à l'ATP, un domaine de liaison du peptide, un domaine Loop, et un domaine C-terminal (adapté d'après Dorard et al., 2011).

2.3. Activité chaperon et fonctions

La surexpression d'HSP110 dans différentes lignées cellulaires augmente leur survie en condition de choc thermique (Oh et al., 1997). La capacité d'HSP110 à conférer cette thermotolérance est dépendante de son activité chaperon. Le choc thermique entraine la déstabilisation des structures tertiaires et quaternaires des protéines, conduisant à l'exposition de surfaces interactives. HSP110 est capable d'empêcher l'agrégation des protéines endommagées en interagissant avec ces surfaces. HSP110 possède ainsi une très forte activité de stabilisation des protéines dénaturées *in vitro*, supérieure à celle d'HSP70 et d'HSC70 (Oh et al., 1999). L'activité anti-agrégation d'HSP110 est plus importante en présence d'ADP, comparativement à celle d'ATP. Celle-ci semble donc être plus efficace en condition de stress sévère, durant lequel le niveau cellulaire d'ATP diminue grandement (Yamagishi et al., 2003). L'implication des différents domaines d'HSP110 dans sa capacité à empêcher l'agrégation a été déterminée par l'utilisation de mutants de délétion (Oh et al., 1999). Son domaine de liaison de l'ATP, tout comme celui d'HSP70, n'est pas ainsi nécessaire pour empêcher l'agrégation (Freeman et al., 1995). Les mutants ne possédant pas le domaine C-terminal sont en revanche non fonctionnels, celui-ci est donc, avec le domaine de liaison au peptide, nécessaire à sa capacité de stabilisation. Le domaine Loop, quant à lui, bien que non essentiel, influence ses

fonctions anti-agrégations, sa délétion diminuant en effet grandement sa capacité de stabilisation (Oh et al., 1999). De retour en condition physiologique, HSP110 peut alors replier les protéines dénaturées en collaboration avec HSP40, avec consommation d'ATP (Mattoo et al., 2013).

HSP110 possède également des fonctions anti-apoptotiques. HSP110 est ainsi capable de supprimer l'activation des caspases 3 et 9 en empêchant la translocation de Bax à la mitochondrie et la libération du cytochrome C (Yamagishi et al., 2006). HSP110 inhibe également l'apoptose en supprimant la signalisation p38 MAPK (Yamagishi et al., 2008).

HSP110 peut finalement fonctionner comme une chaperonne d'ARN. HSP110, tout comme HSP70, est capable de lier *in vitro* certaines régions de l'ARNm, et notamment les régions riches en acides ribonucléiques adénine et uracile (A et U) de différentes cytokines (Il-2, Il-10, IFNγ,…). Ces séquences sont localisées en 3' des ARNm et sont impliquées dans leur stabilité. HSP110 pourrait donc ainsi réguler leur translocation, leur dégradation et leur traduction. Le rôle des HSP dans ce domaine est à ce jour très peu connu (Henics et al., 1999).

2.4. Interactions d'HSP110 avec les autres HSP

Les protéines de choc thermique intracellulaires forment un réseau de chaperons interagissant avec les polypeptides nouvellement synthétisés et dénaturés. HSP110 peut ainsi interagir avec d'autres chaperonnes et existe dans les cellules sous la forme de complexes protéiques dont la taille est comprise entre 400 et 700 kDa (Hatayama et al., 1998; Wang et al., 2000). HSP110 peut ainsi interagir directement avec HSC70 et HSP25 dans différents types cellulaires (Oh et al., 1997; Wang et al., 2000). Ces complexes peuvent être reconstitués en utilisant des protéines purifiées et se forment en absence de substrat. Quand un substrat, comme la luciférase, est ajouté à ce système, celui-ci peut s'associer au complexe suite à un choc thermique, confirmant l'activité de chaperon du complexe (Wang et al., 2000).

De la même manière, Sse1, l'homologue d'HSP110 chez la levure, existe sous la forme de complexes stables avec Ssa et Ssb, les deux HSP70 cytosoliques de levure (Shaner et al., 2005; Yam et al., 2005). Sse1 peut se lier à l'HSP70 de levure et de mammifère dans une stœchiométrie 1:1 avec une forte affinité (Dragovic et al., 2006; Raviol et al., 2006; Shaner et al., 2006). Chez la levure, les autres protéines modulant le complexe Sse1/Ssa, telles que Ydj1,

l'homologue d'HSP40, ne sont pas trouvées dans les complexes natifs et ne sont pas nécessaire pour l'interaction *in vitro* (Shaner et al., 2005; Yam et al., 2005).

2.4.1. L'activité de facteur d'échange de nucléotide d'HSP110 (NEF)

La famille HSP70 est une famille de protéines chaperons possédant une faible activité adénosine triphosphatase. HSP70 alterne entre deux états, un état de faible, et un état de forte affinité pour la liaison du substrat en fonction de l'hydrolyse d'ATP. L'HSP70 liée à l'ATP a ainsi une faible affinité pour le substrat. L'HSP70 liée à l'ADP a quant à elle une forte affinité pour celui-ci (Schmid et al., 1994). Plusieurs protéines, la plupart étant des chaperonnes elles-mêmes, peuvent moduler les fonctions d'HSP70 à travers la régulation de son cycle de l'ATP ou en ciblant son substrat.

HSP40, également appelée DnaJ, est ainsi capable de se lier transitoirement à HSP70 afin d'accélérer son hydrolyse de l'ATP et donc améliorer son activité (Cyr et al., 1992). Le domaine J, d'environ 70 acides aminés et localisé dans la partie N-terminale d'HSP40, est présent dans plusieurs protéines non chaperonnes. Ce domaine permet le recrutement d'HSP70 et la régulation par celle-ci de différents processus biologiques (Cheetham and Caplan, 1998).

Les protéines BAG1 et HspBP1 participent quant à elles à l'autre moitié du cycle de l'ATP d'HSP70, en facilitant l'échange de l'ADP par l'ATP (Hohfeld and Jentsch, 1997; Kabani et al., 2002a; Kabani et al., 2002b; Shomura et al., 2005). Ces protéines sont qualifiées de facteurs d'échange de nucléotides (NEF). Deux mécanismes permettant la libération de l'ADP d'HSP70 ont ainsi été caractérisés. Le domaine de liaison du nucléotide (NBD) d'HSP70 est composé de deux lobes encadrant une profonde poche de liaison du nucléotide (Flaherty et al., 1990). BAG1 peut entraîner la libération de l'ADP en induisant un changement conformationnel dans le second lobe avec peu de changements ailleurs dans le domaine (Sondermann et al., 2001). HspBP1 induit, quant à elle, des déformations importantes entre les deux lobes du domaine de liaison du nucléotide suite à sa liaison sur le second lobe. (Shomura et al., 2005).

L'implication d'HSP110 dans la régulation d'HSP70 a principalement été mise en évidence chez la levure, où il a été observé que la présence de Sse1 stimulait l'activité des HSP70 cytosoliques de levure et de mammifère. Ces résultats ont ensuite été confirmés pour

l'HSP110 de mammifère (Dragovic et al., 2006; Raviol et al., 2006; Shaner et al., 2006). HSP110 est ainsi capable de réguler les fonctions des protéines de la famille d'HSP70 à travers son activité de facteur d'échange de nucléotide (NEF). HSP110 peut interagir stablement avec HSP70 dans différents compartiments cellulaires afin de réguler ses fonctions, en accélérant l'échange de nucléotide et en modulant les activités cellulaires d'HSP70. Le mécanisme d'action d'HSP110 dans la régulation d'HSP70 est proche de celui de la protéine BAG-1 et pourrait se dérouler en trois étapes (Figure 5) (Andreasson et al., 2008).

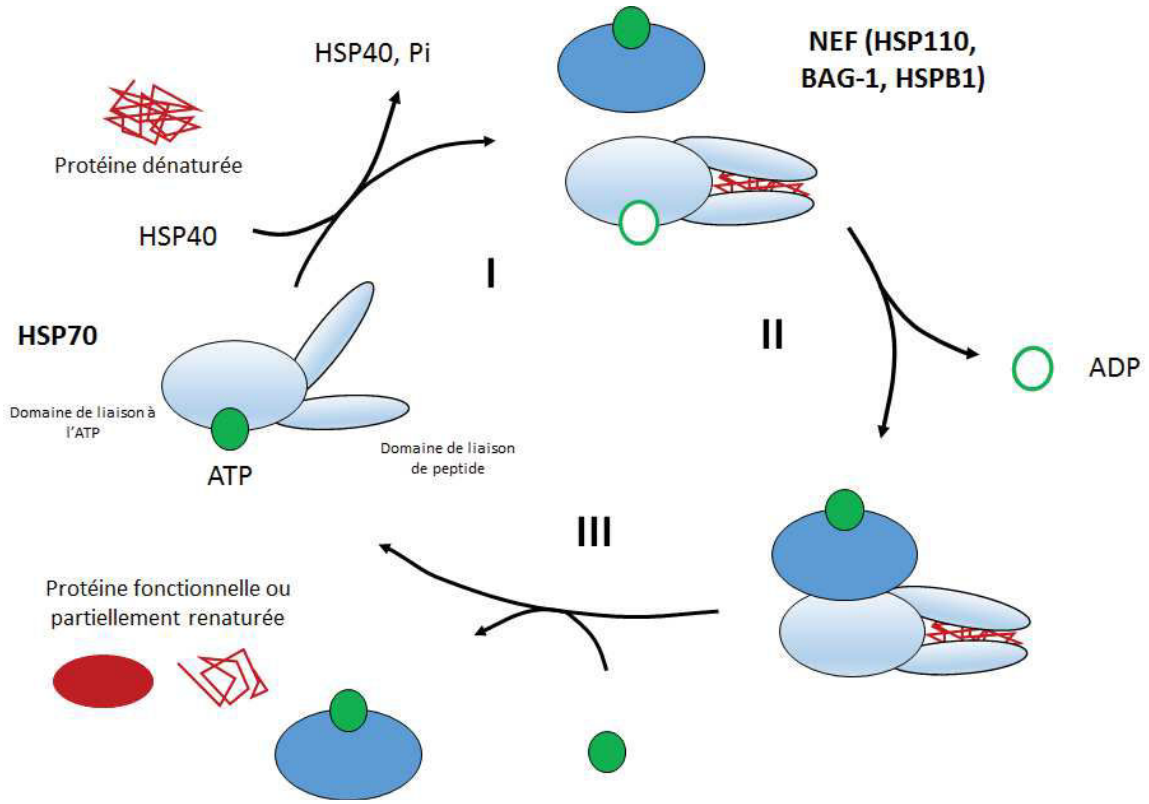

Figure 5 : Modèle pour la coopération entre HSP110 et HSP70 dans le repliement de protéines natives ou dénaturées.
Afin de réguler la fonction d'HSP70, d'autres HSP, notamment HSP40 et HSP110, doivent intervenir et agissent au niveau du domaine de liaison à l'ATP (adapté d'après Polier et al., 2008).

1ere étape : La liaison d'un substrat et d'une protéine contenant un domaine J (telle que HSP40) déclenchent l'hydrolyse de l'ATP liée à HSP70 (Mayer and Bukau, 2005). Cette étape est l'étape limitante de l'échange de nucléotide dans la réaction. (Shaner et al., 2005; Steel et al., 2004). L'hydrolyse de l'ATP entraine des changements conformationnels, la fermeture du domaine de liaison du peptide d'HSP70 et une interaction plus étroite avec le substrat.

2eme étape : L'interaction d'HSP110 avec HSP70 entraîne le relargage de l'ADP lié à HSP70. Les NBD d'HSP70 et d'HSP110 interagissent ensemble. HSP110 pourrait interagir également directement avec le substrat, mais l'éventuelle contribution du domaine de liaison du substrat d'HSP110 dans le repliement n'est pas connue. La liaison de l'ATP à HSP110, mais pas à HSP70 semble nécessaire pour l'heterodimerisation des deux protéines, comme semble l'indiquer l'utilisation de mutants dans le domaine de liaison à l'ATP de Sse1 (Dragovic et al., 2006; Raviol et al., 2006; Shaner et al., 2006). Le domaine de liaison du substrat de Sse/HSP110 est également nécessaire pour positionner correctement les deux NBD. La troncature de la partie C terminale supprime l'activité de NEF (Dragovic et al., 2006; Shaner et al., 2006).

3eme étape : La liaison d'ATP à HSP70 pourrait déclencher la dissociation d'HSP110 du complexe substrat/HSP70/ATP et la dissociation du substrat d'HSP70 en permettant un repliement partiel ou total de la protéine. Les protéines repliées de façon incomplète pourrait subir un nouveau cycle de repliement.

2.4.2. L'activité désagrégase d'HSP110

Les protéines chaperons bloquent l'agrégation des protéines et les aident activement à atteindre leur conformation native. La dénaturation des protéines peut cependant saturer ces systèmes, surtout en condition de stress, en raison du vieillissement et/ou d'une maladie. La dénaturation des protéines peut conduire à différentes maladies neurodégénératives telles que les maladies d'Alzheimer, de Parkinson, de Creutzfeldt Jakob et de Huntington (Cushman et al., 2010; Jackrel and Shorter, 2011)

Une nouvelle HSP, HSP104, a été découverte chez *Saccharomyces cerevisiae*. Celle-ci joue un rôle clef en permettant aux cellules de survivre en condition de stress sévère. Hsp104 est ainsi capable de solubiliser les agrégats protéiques résultants de ce stress et de permettre le recouvrement de protéines enzymatiques actives. HSP104 peut désagréger de nombreuses structures, telles que les amyloïdes stables et les agrégats désordonnés. HSP104 agit seul mais peut aussi coopérer avec d'autres chaperonnes telles qu'HSP70, HSP40 et les petites HSP. HSP104 est très conservée chez les eubactéries et les levures. HSP104 ne montre cependant pas d'homologue chez les métazoaires (Shorter, 2008).

Les mammifères possèdent également un système de désagrégation des protéines. Celui-ci est composé des protéines HSP110, HSP70 et HSP40. La combinaison de ces trois protéines

est ainsi capable d'établir une activité désagrégase et de replier les protéines à partir de larges agrégats dénaturés dans le cytosol des cellules de mammifère. HSP110 semble agir principalement en tant que NEF d'HSP70 pour cette activité. L'activité désagrégase est conservée chez les homologues de la levure. Ainsi, Sse1, Ssa1 et Ydj1 peuvent protéger les protéines de l'agrégation. Cette activité est cependant plus lente en comparaison à celle d'HSP104 (Rampelt et al., 2012; Shorter, 2011).

Les protéines mal repliées dans la cellule peuvent former des amyloïdes et des prions. Les amyloïdes sont des protéines formant des fibres longues et stables, s'allongeant à leurs extrémités en convertissant d'autre copies de la protéine initiale. Quand les fibres amyloïdes deviennent infectieuses, elles sont appelées prions (Cushman et al., 2010; Eisenberg and Jucker, 2012; Shorter, 2010). L'activité désagrégase d'HSP110, d'HSP70 et d'HSP40 n'est cependant efficace que sur les agrégats protéiques amorphes (Shorter, 2011). Celles-ci nécessitent la collaboration des petites HSP pour dépolymériser très lentement les amyloïdes et prions à partir de leurs extrémités (Carulla et al., 2005; Carulla et al., 2010; Duennwald et al., 2012). Le mécanisme d'action est mal connu, mais ces HSP pourraient agir en accélérant la dissociation de monomères d'amyloïdes ou en empêchant la réassociation de monomères libérés des fibres. (Duennwald et al., 2012).

2.5. Expression d'HSP110 dans les cancers

La protéine HSP110 est surexprimée dans de nombreux cancers, tels que les mélanomes, les cancers mammaires et de la prostate, les cancers pancréatiques et colorectaux (Kai et al., 2003; Muchemwa et al., 2008; Muchemwa et al., 2006; Park et al., 2009). L'expression d'HSP110 est associée dans le cancer colorectal au caractère agressif de la tumeur. Son expression est associée à la progression tumorale, à la présence de métastases au niveau des ganglions lymphatiques et à un mauvais pronostic pour les patients (Hwang et al., 2003; Slaby et al., 2009). HSP110 est retrouvée dans le cytoplasme et à la membrane des lymphomes B non-hodgkiniens, son expression est également associée à l'agressivité de ce type de cancer (Zappasodi et al., 2011).

Les fonctions d'HSP110 dans le cancer sont cependant peu connues. HSP110 participe tout d'abord à la survie des cellules grâce à ses propriétés anti-agrégations et anti-apoptotiques, contribuant ainsi à l'augmentation de la résistance des cellules cancéreuses à la chimiothérapie

(Yamagishi et al., 2006; Yamagishi et al., 2008). Les cancers colorectaux de type MSI exprimant faiblement HSP110 montrent ainsi une meilleure réponse à la chimiothérapie (Collura et al., 2014; Kim et al., 2014). La déplétion d'HSP110 montre que cette protéine est essentielle à la survie de différents cancers, notamment colorectaux et gastriques. L'inhibition de son expression entraîne l'apoptose des cellules cancéreuses, par un mécanisme faisant intervenir les caspases. Sa déplétion n'a en revanche aucun effet sur l'apoptose dans des fibroblastes sains (Hosaka et al., 2006).

HSP110 est également impliquée dans la prolifération des cellules cancéreuses. La voie de signalisation Wnt/β-caténine joue un rôle majeur dans le cancer colorectal. HSP110 appartient au complexe de dégradation de la β-caténine et peut recruter la phosphatase PP2A afin de déphosphoryler la β-caténine et éviter sa dégradation (Yu et al., 2015). La déplétion d'HSP110 dans les lymphomes B entraîne une diminution de la croissance tumorale. HSP110 agit dans ses cancers en stabilisant, par interaction directe, les protéines c-Myc et Bcl-6, deux protéines oncogéniques clef dans ces cancers (Zappasodi et al., 2015).

Chapitre II. Le cancer colorectal

Le cancer colorectal (CRC) est une cause majeure de mortalité dans le monde. En 2012, en France, le cancer colorectal était le troisième cancer le plus fréquent chez l'homme et le second chez la femme. Il touche environ 42000 personnes par an et entraine environ 18000 décès. Le taux d'incidence est de 38,4 pour l'homme et de 23,7 pour la femme pour 100000 habitants.

Environ 80% des cancers colorectaux sont d'origine sporadique. L'âge est en effet le principal facteur de risque, avec plus de 90% des CRC se produisant chez des individus après 50 ans (Cappell, 2008). L'incidence du cancer colorectal est plus importante dans les pays développés et peut s'expliquer par le mode de vie. Différents facteurs environnementaux sont ainsi impliqués dans l'augmentation des risques de CRC, tels que l'alimentation, l'alcool, la cigarette, le manque d'activité physique, l'obésité et le diabète (Wei et al., 2009).

Des prédispositions génétiques peuvent également favoriser le CRC. Approximativement 5% des CRC sont dus à des mutations de gènes hérités (Penegar et al., 2007). Environ 20% des patients atteints de CRC présentent un historique familial mais ne peuvent pas être catégorisés dans les syndromes de CRC héréditaires connus (Power et al., 2010). Les syndromes héréditaires de CRC les plus fréquents sont la polypose adénomateuse familiale (PAF) et le syndrome de Lynch, également appelé le cancer du côlon héréditaire non polyposique (HNPCC). La PAF est due à des mutations génétiques dans le gène *APC* (Groden et al., 1991). Elle représente environ 1% de tous les CRC. Les patients atteints de la PAF développent des centaines à des milliers de polypes adénomateux colorectaux. Le risque d'être atteint d'un CRC pour ces patients est proche de 100% (Half et al., 2009).. Le syndrome de Lynch représente quant à lui 2 à 3% des CRC. Ce syndrome est dû à des mutations génétiques dans une des protéines impliquée dans un gène du système de réparation des mésappariement de l'ADN (MMR), entrainant des erreurs lors de la réplication et un risque plus élevé de CRC (Desai and Barkel, 2008). Ce syndrome a permis de mettre en évidence l'instabilité microsatellitaire (MSI) comme voie de tumorigenèse (Brosens et al., 2015). Les maladies inflammatoires de l'intestin, comme la maladie de Crohn, peuvent également faciliter l'établissement d'un CRC (Kalla et al., 2014).

Le développement d'un cancer colorectal est la conséquence d'une accumulation progressive d'altérations génétiques et épigénétiques, conduisant à la transformation de cellules normales de la muqueuse du colon en un cancer invasif. Ces altérations génétiques et épigénétiques provoquent des dérégulations dans les voies de signalisation cellulaires impliquées dans le métabolisme, la prolifération, la survie, et l'apoptose. Les mutations de différents gènes ont été associées à la carcinogenèse colorectale mais le rôle exact d'un grand nombre d'entre eux dans l'initiation et la progression de la maladie est à déterminer (Starr et al., 2009). La transformation d'un adénome en cancer prend généralement 10 à 15 ans, donnant aux médecins une fenêtre d'opportunités pour dépister et retirer les lésions pré malignes ou malignes.

Plusieurs voies moléculaires de la tumorigenèse du cancer colorectal ont été identifiées et mettent en évidence la nature hétérogène du CRC. Le premier modèle de développement tumoral du CRC a été proposé par Fearon et Vogelstein et repose sur l'instabilité chromosomique (Fearon and Vogelstein, 1990). Deux autres voies moléculaires de développement du CRC ont été ensuite décrites. La première repose sur l'instabilité des microsatellites (MSI), causée par des mutations dans les gènes MMR (Smaglo and Marshall, 2013). La deuxième voie, appelée CpG Island Methylator Phenotype (CIMP), repose sur la découverte du rôle de l'épigénétique, en particulier de l'hypermethylation de nucléotides dans la région promotrice de gènes (Nazemalhosseini Mojarad et al., 2013). Ces trois voies ne sont pas forcément exclusives entre elles.

1. Les différentes voies de tumorigenèse du cancer colorectal

1.1. L'instabilité chromosomique (CIN)

L'instabilité chromosomique est la cause la plus fréquente de l'instabilité génomique dans le CRC, et représente environ 75 à 80% des CRC sporadiques. Elle est caractérisée par le gain ou la perte de chromosomes entiers ou de régions chromosomiques hébergeant des gènes nécessaires au processus de cancérogenèse (aneuploïdie), et par une fréquence élevée de perte d'hétérozygotie. L'instabilité chromosomique résulte d'une mauvaise ségrégation des chromosomes au cours de la mitose, d'un dysfonctionnement des télomères, ou de défauts dans la réponse aux dommages de l'ADN. (Pino and Chung, 2010). Les amplifications sur les chromosomes 7, 8q, 13q, 20, et X, et les délétions sur les chromosomes 1, 4, 5, 8p, 14q, 15q,

17p, 18, 20p et 22q sont les plus fréquentes. Les gains ou pertes de chromosomes comprennent des régions contenant des gènes importants pour le développement du cancer tels que le *VEGF, MYC, MET, LYN, PTEN* (Sheffer et al., 2009). Les chromosomes 1, 5, 8, 17 et 18 présentent la fréquence la plus élevée de perte d'allèle (46-78%). La perte d'un chromosome entier est plus fréquente pour le chromosome 18 (Thiagalingam et al., 2001).

La progression du cancer colorectal suivant la voie de l'instabilité chromosomique suit le modèle proposé par Fearon et Vogelstein (Fearon and Vogelstein, 1990). En plus des différentes anomalies du caryotype, on peut retrouver une accumulation de mutations dans les oncogènes et les gènes suppresseurs de tumeurs. Les altérations génétiques ponctuelles les plus courantes concernent les gènes *APC, TP53* et *KRAS*. Celles-ci peuvent être associées aux stades de développement morphologiques du CRC (Figure 6).

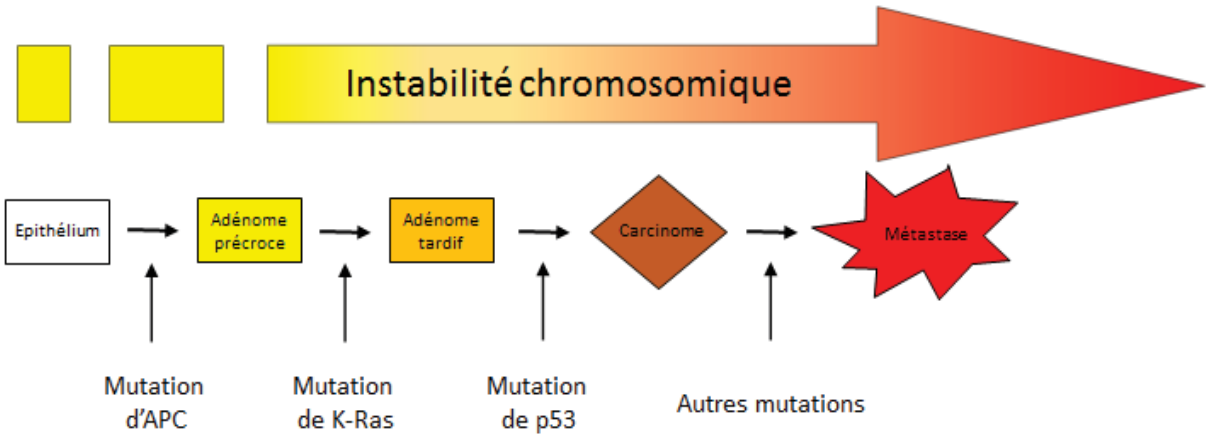

Figure 6 : Modèle de Fearon et Volgelstein.
La transformation d'adénome colorectaux en carcinome est un processus à plusieurs étapes nécessitant la mutation de gènes suppresseurs de tumeur tels que *APC* et *TP53*, et d'oncogènes tels que *KRAS*. (Adapté de Moran et al 2010)

Le gène *APC*, localisé sur le chromosome 5q21, est muté dans 60% à 80% des CRC, ainsi que dans un grand pourcentage des lésions précurseurs (adénomes colorectaux). La perte allélique du chromosome 5q est retrouvée dans 20 à 50% des CRC sporadiques. L'inactivation d'APC est un événement précoce dans le processus de tumorigenèse colorectale. La perte de fonction des deux allèles est nécessaire pour l'inactivation d'APC (Powell et al., 1992). La protéine APC est impliquée dans la régulation du renouvellement des cellules épithéliales du colon (Fevr et al., 2007) et appartient à la voie de signalisation canonique Wnt (Figure 7).

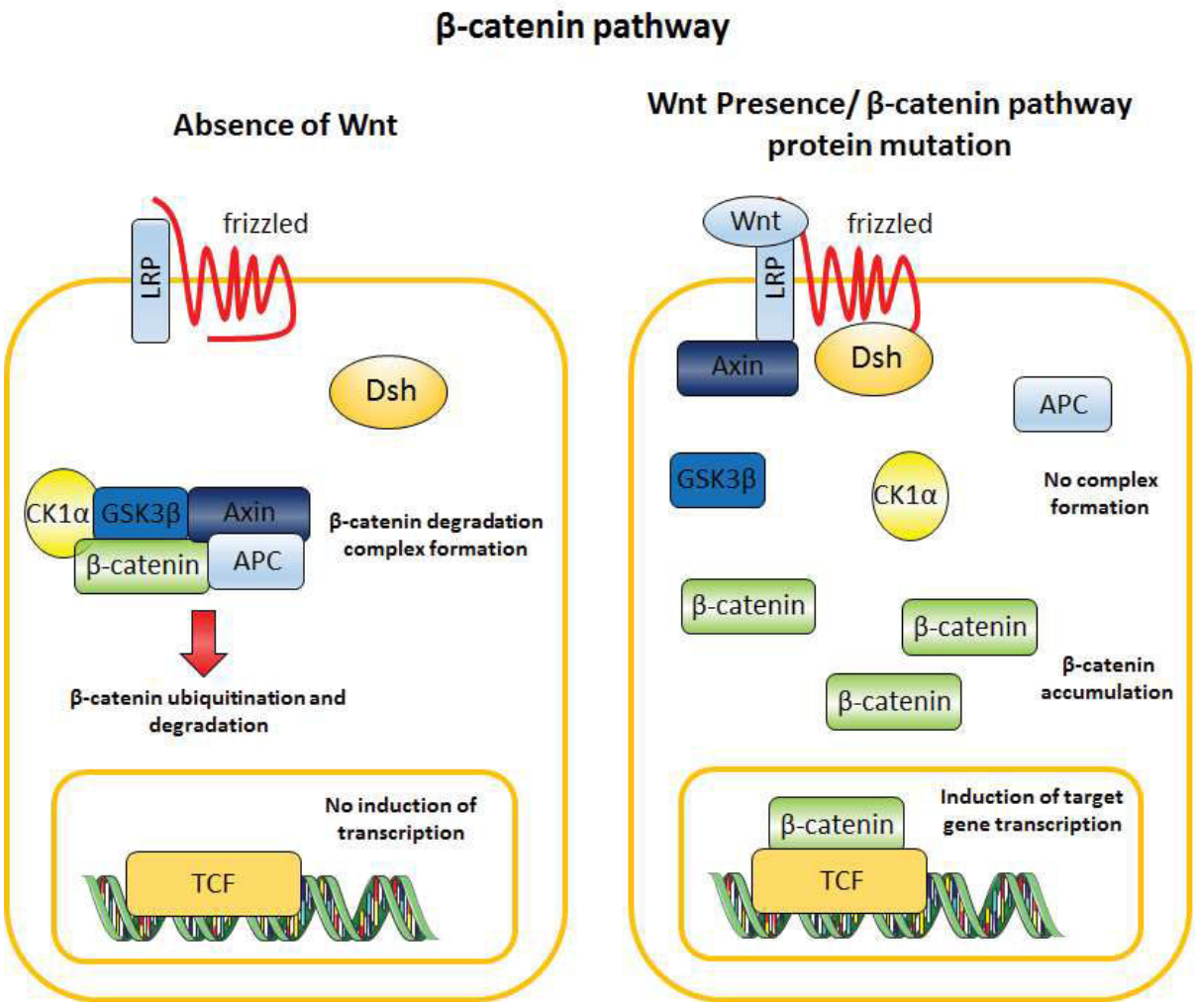

Figure 7 : La voie de signalisation Wnt/β-caténine.
La liaison de Wnt à un récepteur Frizzled entraine l'activation de la protéine Dsh, bloquant l'activation du complexe de destruction de la β-caténine, comprenant l'axine, APC, la caséine kinase 1α et la GSK3β. En absence de Wnt, le complexe de destruction entraîne la phosphorylation de la β-caténine par la GSK3β. La β-caténine est alors ubiquitinée et dégradée par le protéasome. En présence de Wnt ou après des mutations d'APC, la phosphorylation et la dégradation de la β-caténine sont bloquées, permettant son entrée dans le noyau où elle forme des complexes avec TCF et l'induction des gènes cibles de la voie Wnt. (Al-Sohaily et al., 2012)

APC forme un complexe avec la β-caténine, l'axine, et la GSK3β (Kolligs et al., 2002). Dans sa forme non mutée, APC se lie à la β-caténine et induit sa dégradation, en agissant comme un régulateur négatif de la voie de signalisation. L'inactivation fonctionnelle d'APC (par mutation, perte d'hétérozygocité, ou par méthylation de son promoteur) entraîne une accumulation de la β-caténine cytoplasmique, conduisant à sa translocation nucléaire et à sa liaison aux facteurs de transcription TCF/LEF. Les gènes cibles de la voie Wnt affectent de multiples fonctions cellulaires, tels que la prolifération (c-Myc et cycline D1), l'angiogenèse, et

l'apoptose (Behrens, 2005). La voie de signalisation Wnt joue ainsi un rôle important dans l'initiation et la progression du CRC. L'activation de la voie Wnt peut également être déclenchée par des mutations activatrices de la β-caténine, la rendant résistante à la dégradation, par des mutations des gènes *AXIN1* ou *AXIN2*, impliqués eux aussi dans la dégradation de la β-caténine, ou par des mutations activatrices du facteur de transcription TCF-4 (Segditsas and Tomlinson, 2006).

La protéine k-Ras est quant à elle mutée dans 30 à 60% des CRC et dans les adénomes avancés (Pajkos et al., 2000). Le gène *k-Ras* est localisé sur le chromosome 12p12.1 et code pour une protéine de 21kDa liée à la membrane et impliquée dans la transduction du signal. La protéine k-Ras est activée en réponse à différents signaux extracellulaires. La mutation de la protéine bloque la protéine sous sa forme active, en inhibant son activité GTPase. L'activation de k-Ras affecte différentes voies de signalisation impliquées dans la prolifération, la différentiation et l'apoptose (Takayama et al., 2006).

Le gène *TP53*, codant pour p53, est localisé sur le chromosome 17p13.1. La perte de l'allèle 17p est retrouvée dans 50 à 75% des CRC, mais pas dans les adénomes, et semble donc être un événement tardif dans le processus de tumorigenèse colorectal. La perte de l'allèle 17p est souvent associée à des mutations dans le gène *TP53* situé sur le second allèle (Leslie et al., 2002). La protéine p53 est un facteur de transcription capable d'induire l'arrêt du cycle cellulaire en phase G1 afin de faciliter la réparation de l'ADN au cours de la réplication de cellules exposées à des stress environnementaux ou oncogéniques (Takayama et al., 2006). Quand les dommages à l'ADN sont trop importants pour être réparés, p53 peut alors agir comme un gène suppresseur de tumeur en induisant l'apoptose (Pietsch et al., 2008).

1.2. L'instabilité des microsatellites (MSI)

Les microsatellites sont des petites séquences nucléotidiques répétées répandues dans tout le génome. Des erreurs peuvent se produire lors de la réplication de ces séquences en raison de leur répétitivité. Le système de réparation des mésappariement de l'ADN (MMR), composé des protéines MSH2, MSH3, MSH6, MLH1, MLH3, PMS1, PMS2 et EXO1, est capable de reconnaitre et de réparer les paires de bases mal appariées (Figure 8). L'instabilité des microsatellites (MSI) résulte de l'incapacité du système MMR à reconnaitre et corriger ces erreurs.

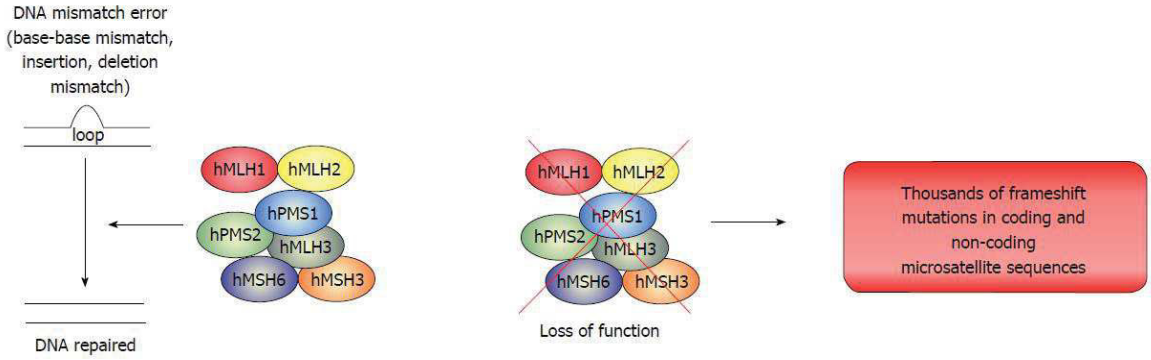

Figure 8 : Le système de réparation des mésappariements de l'ADN (MMR)
Suite à la détection d'une boucle dans l'ADN par un dimère comprenant MSH2/MSH6 (détection des mésappariements, insertion et délétion ponctuelles) ou MSH2/MSH3 (détection des grands mésappariements), le complexe composé de MLH1 et PMS2 est recruté et déclenche l'excision de la boucle à travers le recrutement des protéines PCNA, RFC et Exo1. PMS1 et MLH3 peuvent également se dimériser avec MLH1 mais le rôle de ces dimères est moins bien connu. L'ADN a alors resynthétiser grâce à l'ADN polymérase δ et l'ADN ligase 1 (Moran et al., 2010).

La découverte de l'instabilité des microsatellites en 1993, et son lien avec le syndrome de Lynch, ont entrainé la reconnaissance de la voie MSI comme une voie alternative dans la tumorigenèse du cancer colorectal. La mutation germinale dans les gènes du système MMR conduit au syndrome de Lynch. La mutation somatique ou l'extinction de l'expression par hypermethylation des gènes MMR représentent quant à elles environ 15% des CRC sporadiques. Le cancer colorectal sporadique hautement MSI est généralement provoqué par l'extinction de l'expression du gène *MLH1* suite à l'hypermethylation de son promoteur (Veigl et al., 1998).

Les tumeurs hautement MSI sporadiques sont plus fréquentes chez les femmes âgées, et localisées de façon prédominante dans le colon droit. Les tumeurs MSI sont majoritairement diploïdes, d'une histologie mucineuse, peu differenciées et présentent peu de mutations dans les gènes *KRAS* et *TP53*. Les tumeurs hautement MSI montrent une forte résistance à différents agents chimiothérapeutiques, tels que le 5-FU et le cisplatine (Aebi et al., 1996; Warusavitarne et al., 2006). Le phénotype hautement MSI est cependant associé à une meilleure survie en comparaison avec les patients MSS (Guastadisegni et al., 2010). Ces tumeurs présentent une forte infiltration de lymphocytes T CD4+ et CD8+. La forte infiltration lymphocytaire pourrait s'expliquer par le grand nombre de protéines mutées dans ce type de cancer et qui constitueraient autant d'antigènes de tumeurs (Iacopetta et al., 2010).

L'instabilité microsatellitaire conduit en effet à des changements dans le cadre de lecture au niveau des répétitions de microsatellites présents dans les séquences exoniques. Ces changements entraînent alors des mutations dans la séquence protéique et l'apparition d'un codon stop prématuré (Boland and Goel, 2010). L'instabilité microsatellitaire, lorsqu'elle se produit dans un intron, peut conduire au « skipping » de l'exon suivant, et éventuellement à l'apparition d'un codon stop prématuré.

Un grand nombre de gènes, impliqués dans différentes fonctions cellulaires peuvent ainsi être mutés lorsque le système MMR est déficient (Iacopetta et al., 2010). On retrouve parmi ceux-ci des gènes impliqués dans la réparation de l'ADN (*RAD50, MSH3, MSH6, BLM, MBD4, MLH3*), dans l'apoptose (*APAF1, BAX, BCL-10, Caspase 5*), dans la transduction du signal (*TGFβRII, ACTRII, IGFIIR, WISP-3*), dans le cycle cellulaire (*PTEN, RIZ*) mais aussi des facteurs de transcription tels que TCF-4 (Figure 9).

Gene	Mononucleotide coding repeat	Frequency ofmutation (%)
DNA repair		
RAD50	(A) 9	28
MSH3	(A) 8	38
MSH6	(C) 8	22
BLM	(A) 9	9
Apoptosis		
HSP110	**(T) 17**	**100**
APAF1	(A) 10	13
BAX	(G) 8	45
BCL10	(A) 8	13
Caspase-5	(A) 10	48
Signal transduction		
TGFbRII	(A) 10	81
ACTRII	(A) 8	58
IGFIIR	(G) 8	17
WISP-3	(A) 9	31
Cell cycle		
PTEN	(A) 6	18
RIZ	(A) 8, (A) 9	27
Transcription factor		
TCF-4	(A) 9	39

Figure 9 : Liste de plusieurs gènes contenant des séquences codantes répétées pouvant être la cible de mutations dans le cancer colorectal avec instabilité microsatellitaire.
(Adapté d'après Iaocopetta et al., 2010)

La mutation dans le microsatellite poly(A) du *TGFβRII*, avec celle située dans l'intron 8 d'HSP110 (voir partie 3), est l'une des plus fréquemment observée. Elle est retrouvée en effet dans environ 80% des CRC de type MSI (Parsons et al., 1995). Cette mutation inactive la

fonction du gène en conduisant à la production d'un récepteur tronqué (Markowitz et al., 1995). La signalisation du TGF-β2 inhibant la prolifération cellulaire, des altérations dans le gène du récepteur de cette voie peuvent donc contribuer au développement des cancers colorectaux MSI.

La voie de tumorigenèse MSI peut être identifiée suite à l'analyse de cinq séquences microsatellitaires. Ce panel comprenait initialement 2 répétitions de mononucléotide (BAT25 et BAT26) et 3 répétitions de dinucléotides (D5S346, D2S123 et D17S250) (Boland et al., 1998). Il a ensuite été proposé l'analyse de 5 marqueurs mononucléotidiques répétés (BAT25, BAT26, NR21, NR24 et NR27), ces séquences étant plus sensibles et plus spécifiques (Nardon et al., 2010). Les CRC hautement MSI sont définis par l'instabilité d'au moins 2 marqueurs. Les CRC faiblement MSI ne présentent quant à eux qu'un seul marqueur d'instabilité. Les CRC microsatellite stable (MSS) sont ceux qui ne présentent apparemment pas d'instabilités.

L'altération élevée de microsatellites tétranucleotidiques (EMAST) est une autre forme de MSI retrouvée dans environ 60% des CRC. Les CRC faiblement MSI et ceux présentant une EMAST pourraient être dus à la diminution de l'expression du gène *MSH3*, entraînant l'instabilité des dinucléotides et des tétranucleotides (Carethers et al., 2015).

1.3. Le phénotype méthylateur des ilots CpG (CIMP)

Les altérations épigénétiques font référence à des changements dans l'expression de gènes ou de leurs fonctions se produisant sans affecter leur séquence d'ADN. Chez l'homme, les changements épigénétiques sont principalement causés par la méthylation de l'ADN ou la modification des histones. La méthylation de l'ADN se produit sur les dinuléotides 5′-CG-3′ (CpG). La méthylation du la région promotrice d'un gène entraine l'extinction de son expression, pouvant conduire ainsi à la perte de l'expression de gènes suppresseurs de tumeur. L'expression de nombreux gènes impliqués dans la tumorigenèse colorectale, tels que, par exemple, *APC, MCC, MLH1* et *MGMT,* peut être supprimée par l'hypermethylation de l'ADN (Wong et al., 2007). Des facteurs environnementaux tels que la cigarette et l'âge sont corrélés à une méthylation augmentée (Samowitz et al., 2006; Toyota and Issa, 1999).

Cinq séquences ont été choisies pour servir de marqueurs au phénotype méthylateur des îlots CpG et correspondent aux promoteurs des gènes *CACNA1G, IGF2, NEUROG1, RUNX3 et SOCS1*. La positivité au CIMP est définie par la méthylation d'au moins 3 marqueurs

(Weisenberger et al., 2006). Les CRC hautement CIMP représentent environ 20% des CRC sporadiques et ont des caractéristiques distinctes. Les CRC hautement CIMP sont plus fréquents chez les femmes et les personnes âgées. Ces tumeurs sont souvent localisées dans le colon droit, peu différenciées, d'histologie mucineuse, microsatellite instable et mutées sur BRAF (Nosho et al., 2008). Ces patients ne bénéficieraient pas de la chimiothérapie basée sur le 5-FU (Jover et al., 2011).

2. La voie de signalisation STAT3

En plus des voies de tumorigenèse précédemment décrites, l'inflammation joue un rôle important dans l'initiation et le développement des cancers (Grivennikov et al., 2009) et en est désormais le 7ème marqueur (Figure 10) (Colotta et al., 2009).

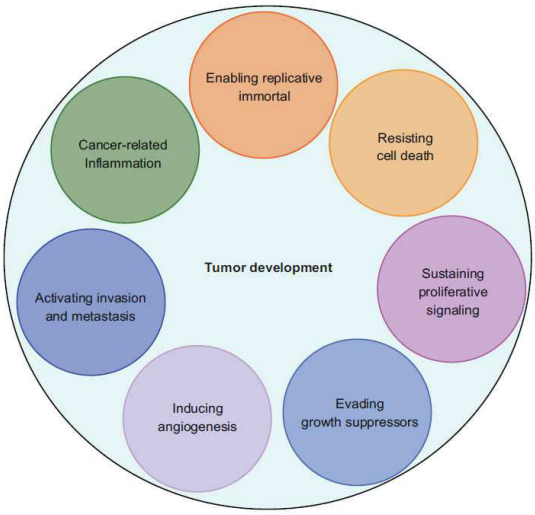

Figure 10 : Les 7 critères nécessaires au développement du cancer.
L'environnement inflammatoire fait désormais partie, au même titre que la capacité à se multiplier de façon infinie, la résistance à l'apoptose, à proliférer, l'insensibilité aux signaux antiprolifératifs, à induire l'angiogenèse et à activer l'invasion et la formation de métastases, des caractéristiques du cancer. (Fan et al., 2013).

La mise en place d'un environnement inflammatoire chronique suite à différentes infections (*Helicobacter pylori*, certains bactéoïdes) est associée au développement de différents cancers tels que les cancers gastriques et du colon (Polk and Peek, 2010; Wu et al., 2009). Des facteurs non infectieux, comme la cigarette et les maladies inflammatoires de l'intestin (syndrome de Crohn, colique ulcérative,..) peuvent également être responsables d'une inflammation chronique et ainsi augmenter le risque de développement d'un CRC (Takahashi et al., 2010; Waldner and Neurath, 2009). L'inflammation peut également promouvoir le développement du cancer à travers la sécrétion de nombreuses cytokines par les cellules immunitaires innées et adaptatives (Grivennikov et al., 2009; Moore et al., 1999).

La voie de signalisation STAT3 est l'une des voies de signalisation majeure permettant de lier l'inflammation au développement du cancer. STAT3 appartient à la famille des STAT comprenant sept membres, à savoir STAT1, STAT2, STAT3, STAT4, STAT5α, STAT5β et STAT6. On peut distinguer principalement deux groupes au sein de cette famille. Les facteurs de transcription STAT2, STAT4 et STAT6 sont activés par un petit nombre de cytokines et sont impliqués dans le développement des lymphocytes T et dans la signalisation de l'IFNγ. STAT1, STAT3 et STAT5 sont quant à eux activés dans différents tissus par de nombreux ligands. Ils sont impliqués dans la signalisation de l'IFNγ, le développement mammaire et l'embryogenèse. Ces derniers jouent un rôle clef dans l'oncogenèse en contrôlant la progression du cycle cellulaire et l'apoptose (Subramaniam et al., 2013).

Le facteur de transcription STAT3 est celui qui a été le plus étudié en raison de son implication dans de nombreuses voies de signalisation oncogéniques, et dans les voies de transduction du signal intracellulaire de plusieurs cytokines pro-inflammatoires et facteurs de croissance. STAT3 est également impliqué dans le développement, la différenciation, l'immunité, le métabolisme et est surexprimé dans différentes maladies telles que les cancers (Levy and Lee, 2002).

2.1. Structure du facteur de transcription STAT3

Le facteur de transcription STAT3 est une protéine de 780 acides aminés et est composé de 6 domaines (Figure 11):

- Le domaine N-terminal est impliqué dans l'oligomérisation des dimères de STAT3 (Kisseleva et al., 2002).
- Le domaine coiled-coil est constitué de 4 hélices α et est impliqué dans l'interaction avec d'autres protéines, comme les facteurs de transcription c-Jun et IRF (Horvath et al., 1996; Kisseleva et al., 2002).
- Le domaine de liaison à l'ADN de STAT3 permet la liaison des homodimères de STAT3 ou hétérodimères STAT1/STAT3 aux séquences consensus présentes sur les promoteurs des gènes cibles. Il est organisé autour d'une structure en feuillets β connectée par des boucles non structurées formant une surface hydrophile et permettant l'interaction avec l'ADN.
- Le domaine « linker » permet de relier le domaine de liaison à l'ADN au domaine SH2 de STAT3.

- Le domaine SH2 forme une poche capable de lier les tyrosines phosphorylées. Cette région est impliquée dans la dimérisation des facteurs de transcription STAT3 et permet aussi la liaison de la protéine aux récepteurs membranaires, principalement les récepteurs de cytokines (famille gp130), qui sont activés et phosphorylés sur une tyrosine dans la partie cytoplasmique (Hemmann et al., 1996; Shuai et al., 1992).
- Le domaine se situant au niveau de l'extrémité C-terminale correspond au domaine d'activation de la transcription et contient deux sites de phosphorylation, le résidu tyrosine 705 et la sérine 727, ainsi qu'un site d'acétylation (la lysine 685).

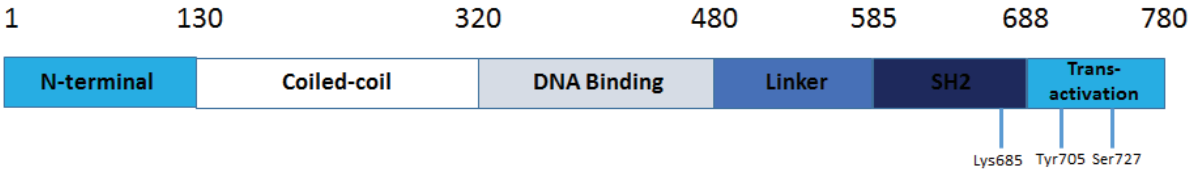

Figure 11 : Structure de la protéine STAT3.
La protéine STAT3 est composée de 6 domaines et comporte deux sites de phosphorylation, sur la tyrosine 705 et la serine 727.

2.2. Mécanisme général d'activation de la voie STAT3

De nombreux ligands peuvent entraîner la phosphorylation de STAT3. Celui-ci est activé en réponse à différents facteurs telles que les interleukines de la famille de l'IL-6 (IL-6, LIF, cardiotrophin-1, CNTF, IL-11, OSM), d'autres cytokines et chemokines comme l'IL-10, l'IL-12, l'Il-22, le TNF-α, l'IFNγ, CCL2, CCL3, et des facteurs de croissance tels que l'EGF, le TGFα et le PDGF. La voie STAT3 peut également être activée par des carcinogènes (cigarette, particules fines de diesel…) et des stress environnementaux (ultraviolet, choc thermique, stress oxydants) (Siveen et al., 2014).

Une grande variété de récepteurs est donc impliquée dans l'activation de la voie STAT3. On retrouve, en plus des récepteurs aux cytokines, les récepteurs aux facteurs de croissance tels que l'EGFR, le FGFR, le PDGFR et le VEGFR (Debnath et al., 2012). Les récepteurs ne possédant pas d'activité tyrosine kinase intrinsèque pour phosphoryler STAT3 recrutent des tyrosines kinases telles que JAK et Src.

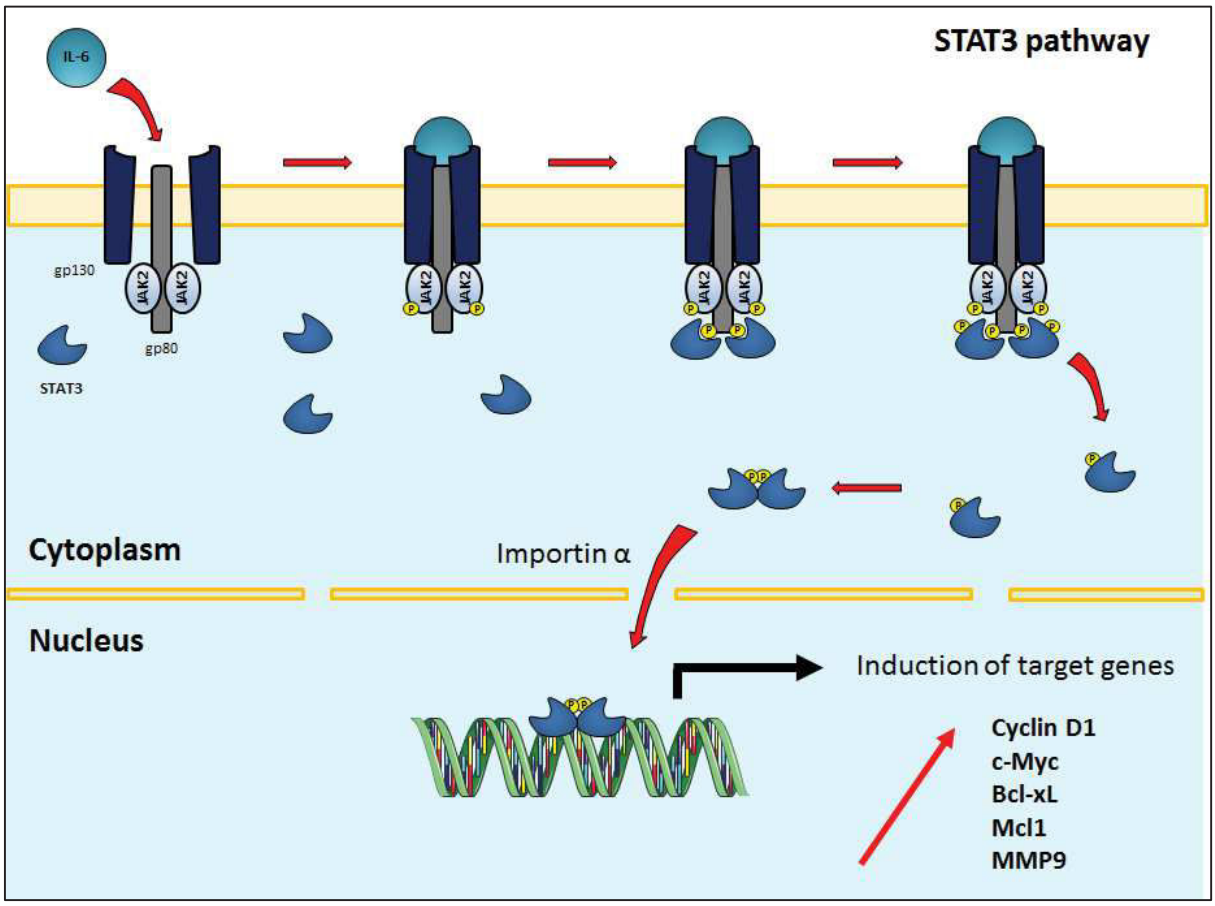

Figure 12 : Mécanisme d'activation de la voie STAT3 par l'IL-6.
Suite à la liaison d'une cytokine à son récepteur, des protéines tyrosines kinase telles que les JAK se lient a ce récepteur et le phosphoryle, permettant le recrutement d'un STAT3 cytoplasmique latent et sa phosphorylation. Les STAT3 phosphorylés peuvent alors se dimériser et entrer dans le noyau où ils induisent l'expression de gènes cibles.

La fixation d'une cytokine sur son récepteur entraîne la dimérisation d'une protéine de transduction du signal à la membrane, la gp130 (Figure 12). Cette dimérisation conduit à la phosphorylation de JAK2, une tyrosine kinase liée à gp130. JAK2 phosphoryle ensuite gp130 sur une tyrosine qui va servir de site d'ancrage à un STAT3 cytoplasmique *via* son domaine SH2. JAK2 peut alors phosphoryler la tyrosine 705 de ce monomère de STAT3. Les monomères phosphorylés se dimérisent et sont pris en charge par l'importine α5 et l'importine α7 pour leur entrée dans le noyau (Ma and Cao, 2006). STAT3 se lie alors à des éléments de réponse de l'ADN spécifiques tels que les éléments de réponse de stimulation à l'IFNγ (ISRE) présents dans les régions promotrices de gènes cibles pour réguler leur transcription. Le dimère de STAT3 reconnait un élément de l'ADN répété inversé de 8 à 10 paires de bases avec une séquence consensus 5'-TT(N)AA-3'. L'activité transcriptionnelle de STAT3 nécessite le

recrutement de coactivateurs, tels que CBP/p300, APE1/Ref-1, et NcoA (Giraud et al., 2002; Gray et al., 2005).

L'activité transcriptionnelle peut être augmentée par la phosphorylation de la serine 727 dans le domaine de transactivation. Cette sérine peut être phosphorylée par différentes kinases, notamment mTOR, Cdk5, PKCδ et PKCε (Aziz et al., 2007; Fu et al., 2004; Jain et al., 1999; Yokogami et al., 2000). STAT3 peut également être acétylé sur le résidu Lysine en position 685 par l'histone acetyltransferase p300 afin de stabiliser les dimères formés (Yuan et al., 2005).

L'activation de la voie STAT3 dans les cellules normales est transitoire. Le pic de phosphorylation de STAT3 se produit entre 15 et 60 minutes après l'exposition à une cytokine, puis diminue constamment sur plusieurs heures, malgré la présence persistante de cytokines. Ce processus est dû à des protéines régulatrices négatives de l'activation, comprenant les protéines de la famille SOCS, la protéine CIS, les protéines inhibitrices des STAT activés (PIAS), différentes tyrosines phosphatases (PTP) telles que SHP-2, PTP1B, PTPεC, TC45, SHP-1 et le système de dégradation des protéines par le protéasome (Masciocchi et al., 2011).

Contrairement aux autres STAT, les STAT3 non phosphorylés peuvent également se dimériser. La navette entre le noyau et le cytoplasme est cependant plus lente. L'interaction entre les deux monomères fait alors intervenir le domaine N-terminal (Delgoffe and Vignali, 2013). Ces dimères de STAT3 non phosphorylé sont capable d'induire la transcription de gènes cibles distincts de ceux des STAT3 phosphorylés (Yang et al., 2007; Yang and Stark, 2008).

2.3. La voie STAT3 dans le cancer

Parmi les différents membres de la famille des STAT, STAT3 est celui qui est le plus souvent corrélé à la tumorigenèse et est considéré comme un oncogène (Bromberg et al., 1999). Contrairement à l'activation transitoire de la voie STAT3 dans les cellules normales, STAT3 est continuellement activé dans de nombreux types de cancer, tels que les lymphomes (Yu et al., 1997), les mélanomes (Niu et al., 2002a), les cancers colorectaux (Corvinus et al., 2005), du pancréas (Wei et al., 2003a) et du poumon (Alexandrow et al., 2012).

L'augmentation de la phosphorylation de STAT3 n'est pas due à des mutations activatrices dans STAT3 mais à l'abondance de facteurs de croissance dans l'environnement tumoral. Différents mécanismes tumorigéniques, tels que l'activation d'oncogènes,

l'inactivation de gènes suppresseurs de tumeur, la dérégulation de l'expression de récepteurs en amont (EGFR,…), ou des mutations activatrices des protéines JAK (Johnston and Grandis, 2011), peuvent déclencher l'activation de la voie STAT3 ou la libération de médiateurs inflammatoires de façon autocrine (Jarnicki et al., 2010). STAT3 peut également être phosphorylé directement dans les cellules cancéreuses par des tyrosines kinases cytoplasmiques appartenant à la famille des SFK telles que Src, Lck, Hck, Lyn, Fyn et Fgr (Silva, 2004). L'activation de STAT3 peut aussi être due à des mutations dans des protéines régulant négativement la voie, comme l'extinction de l'expression de SOCS3 par l'hypermethylation des ilots CpG de son promoteur (He et al., 2003).

STAT3 agit principalement en activant la transcription de gènes cibles impliqués dans la prolifération, la survie, l'angiogenèse et la formation de métastases.

L'activation de la voie STAT3 peut favoriser la croissance tumorale en induisant la transcription de gènes cibles impliqués dans la transition du cycle cellulaire de la phase G1 vers la phase S dans différents cancers et notamment le cancer colorectal (Figure 13) (Bollrath et al., 2009). On retrouve parmi ces gènes cibles la cycline D1, la cycline B, c-Myc, et Cdk1 (Jarnicki et al., 2010). La cycline D1 peut s'associer aux protéines cdk4 et cdk6 et contrôler la progression de la phase G1 à S. Le facteur de transcription c-Myc est, quant à lui, capable d'induire la prolifération à travers la régulation de la transcription de différents acteurs du cycle cellulaire tels que les Cdk, les cyclines, mais aussi les facteurs de transcription E2F (Bretones et al., 2014). Des cellules transformées avec une forme active et constitutivement dimérisée de STAT3 montrent ainsi un niveau de transcription de c-Myc et de la cycline D1 trois à cinq fois supérieur (Bromberg et al., 1999). L'inhibition de la voie STAT3, par des inhibiteurs des protéines JAK ou l'utilisation de siRNA dirigés contre STAT3, inhibe en revanche la croissance tumorale, ainsi que l'expression de la cycline D1 et de c-Myc, dans différents cancers (Zhao et al., 2011).

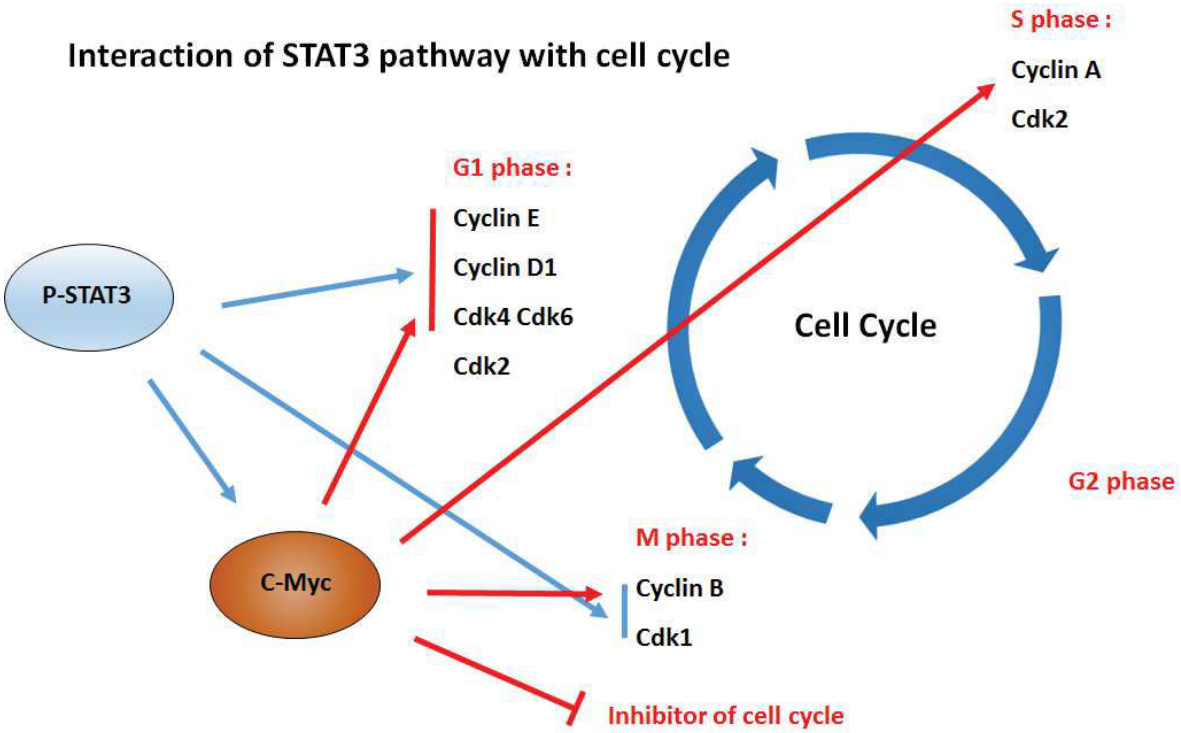

Figure 13 : Interaction de STAT3 avec le cycle cellulaire.
Le cycle cellulaire est un processus qui peut se décomposer en quatre phases. Au cours de la phase G1, les cellules effectuent leur métabolisme normal et se préparent pour la phase suivante, la phase S. Au cours de celle-ci, les cellules répliquent leur ADN. Les cellules vont ensuite continuer à exercer leurs fonctions lors de la phase G2, puis se diviser au cours de la mitose (phase M). La voie STAT3 peut accélérer ces changements de phase en contrôlant l'expression de différentes protéines impliquées dans sa régulation.

La voie de signalisation STAT3 contribue également à la tumorigenèse par l'induction de la transcription de protéines anti-apoptotiques, telles que la survivine, Bcl-xL, Bcl-2, Mcl-1. L'inhibition de la voie STAT3 inhibe l'expression de ces protéines et peut induire l'apoptose (Yu and Jove, 2004). Le facteur de transcription STAT3 favorise de plus la survie des cellules par l'induction de l'expression, en collaboration avec HSF1, de différentes HSP, notamment HSP70, HSP90 et HSP110 (Olszak et al., 2014; Stephanou et al., 1998; Zorzi and Bonvini, 2011).

La voie STAT3 peut favoriser l'angiogenèse. STAT3 peut ainsi réguler l'expression du VEGF dans différents cancers (Niu et al., 2002b; Wei et al., 2003a; Wei et al., 2003b). STAT3 est de plus impliqué dans la régulation de l'expression du facteur de transcription HIF-1α, et ainsi indirectement dans celle du VEGF. STAT3 peut interagir directement avec HIF-1α et être

recruté sur le promoteur du VEGF en condition d'hypoxie (Jung et al., 2005). L'inhibition de STAT3 bloque l'expression d'HIF-1α et du VEGF, inhibant ainsi la croissance tumorale et l'angiogenèse (Xu et al., 2005).

Les gènes cibles de STAT3 comportent finalement différents membres de la famille des MMP (MMP1, MMP2 et MMP9), conférant à cette voie de signalisation des fonctions métastatiques (Dechow et al., 2004; Tsareva et al., 2007; Xie et al., 2004). L'activation de STAT3 corrèle avec les capacités invasives et la formation de métastases dans de nombreux cancers et est associée à un mauvais pronostic (Jarnicki et al., 2010).

3. Expression d'un mutant d'HSP110, HSP110ΔE9, dans les cancers colorectaux de type microsatellite instable

Notre équipe, en collaboration avec celle du Dr Duval (INSERM U938) a identifié HSP110 comme une nouvelle cible de mutation fréquente dans les lignées cellulaires et les tumeurs de cancers colorectaux de type MSI. Cette altération, que l'on peut retrouver sur les deux allèles et dans 100% des tumeurs colorectales primaires MSI, est la mutation la plus fréquente retrouvée dans ce type de cancer par rapport à toutes celles précédemment décrites.

Cette mutation, dans la séquence répétée T17 de l'intron 8 du gène d'HSP110 n'est pas retrouvée dans les lignées et les tumeurs primaires MSS et conduit à l'expression d'une forme tronquée d'HSP110, nommée HSP110ΔE9, en raison du « skipping » de l'exon 9 de la protéine et de l'apparition d'un codon stop prématuré.

Ce mutant est uniquement composé du domaine de liaison à l'ATP de la protéine et agit comme un dominant négatif. Il interagit ainsi dans un ratio 1:1 avec HSP110 et inhibe ses fonctions chaperons et anti-agrégations. Les acides aminés ASP633, GLN707, et GLU708, localisés dans le domaine de liaison du peptide d'HSP110, sont essentiels pour cette interaction (Collura et al., 2014). HSP110ΔE9 est capable de séquestrer HSP110 WT dans le cytoplasme, en inhibant sa localisation nucléaire. Son expression dans des lignées de cancers colorectaux sensibilise les cellules à l'apoptose induite par différentes molécules utilisées en chimiothérapie et diminue la croissance tumorale dans des modèles de xénogreffe de souris.

Les cancers colorectaux de type MSI présentent un meilleur pronostic mais montrent une mauvaise réponse à la chimiothérapie basée sur le 5-FU. De façon intéressante, les patients

avec un fort niveau d'HSP110ΔE9 montrent une meilleure réponse à la chimiothérapie et une meilleure survie à 5 ans (Dorard et al., 2011). Plus la délétion est importante (>4pb) dans le microsatellite d'HSP110, plus l'expression d'HSP110 diminue, alors que celle du mutant est stable. Les patients au stade II ou III avec une délétion supérieure à 5pb ont ainsi une excellente réponse à la chimiothérapie. L'expression d'HSP110ΔE9 chez les patients en stade II ou III et non traité à la chimiothérapie ne présente cependant aucun bénéfice pour la survie. L'effet d'HSP110ΔE9 semble donc uniquement chimio-sensibilisant (Collura et al., 2014). Ces résultats sont également confirmés par une autre équipe, celle-ci montre en effet une association entre le pronostic et l'expression d'HSP110 dans ces tumeurs (Kim et al., 2014).

De façon intéressante, la mutation de l'intron 8 d'HSP110 est associée à la mutation de BRAF V600E. La méthylation du promoteur de MLH1, et la mutation de k-Ras, ne montrent cependant pas de corrélation (Markovic et al., 2013).

Chapitre III. Rôle d'HSP110 dans la différenciation des monocytes en macrophages dans le cancer colorectal

Le développement d'un cancer ne repose pas uniquement sur l'accumulation de mutations génétiques mais est également dépendant de son interaction avec le système immunitaire. Une forte activation du système immunitaire inné et adaptatif peut être observée au cours du développement du cancer colorectal (Galon et al., 2007; Nihon-Yanagi et al., 2012). L'immunosuppression est de ce fait un marqueur important de la progression de nombreux cancers dont le cancer colorectal (Evans et al., 2006).

La théorie de « l'immunoediting », décrivant la réponse du système immunitaire au développement d'une tumeur, a été proposée (Dunn et al., 2004). Celle-ci peut être divisée en trois phases, l'élimination, l'équilibre, et l'échappement. Dans la phase d'élimination, le système immunitaire est capable d'éliminer les cellules tumorales et d'induire une régression tumorale. Dans la phase d'équilibre, le système immunitaire ne réussit pas à éliminer la totalité des cellules tumorales. Certaines cellules sont alors sélectionnées, mutent et deviennent résistantes aux mécanismes de contrôle immunitaire. Cette étape peut prendre plusieurs années. Lors de la phase d'échappement, les cellules tumorales échappent au système immunitaire et peuvent créer un environnement favorable à la progression tumorale.

Les lymphocytes (Deschoolmeester et al., 2010), les macrophages (Forssell et al., 2007) et les cellules dendritiques (Schwaab et al., 2001) sont les cellules immunitaires les plus fréquemment observées dans le microenvironnement tumoral du cancer colorectal. La présence de lymphocytes T et de macrophages est progressivement augmentée au cours de la séquence adénome-carcinome dans le cancer colorectal (Cui et al., 2009).

1. Les principales cellules immunitaires dans le microenvironnement du cancer colorectal

1.1. Les lymphocytes

Une réponse anti-tumorale spécifique peut être générée par le système immunitaire, et en particulier par les lymphocytes T. Les cellules présentatrices d'antigènes (CPA) telles que

les cellules dendritiques et les macrophages, peuvent capturer et présenter les antigènes tumoraux aux cellules T CD4 à travers le CMH de classe II ou aux T CD8 à travers le CMH de type I. L'activation des lymphocytes T nécessite 3 signaux : la reconnaissance d'un peptide antigénique présenté par les CPA, l'activation de molécules co-stimulatrices (CD80/CD28, CD40/CD40L) et le recrutement de cytokines (IL-1, IL-2, IL-6, IL-12, IFNγ). Les lymphocytes T CD8 peuvent reconnaitre et lyser les cellules tumorales. Les cellules T CD4 activées peuvent quant à elles moduler la réponse immunitaire anti-tumorale et se différencier en plusieurs sous-types. Le sous-type Th1 permet la sécrétion de cytokines pro-inflammatoires telles que l'IL-2 et l'IFNγ. Le sous-type Th2 favorise la croissance tumorale. Les sous-types Th17, caractérisés par une forte sécrétion d'IL-17, et Treg, caractérisés par l'expression de CD25 et de Foxp3, inhibe la réponse immunitaire. L'infiltration dans les cancers colorectaux par les lymphocytes T CD8, et notamment par les lymphocytes T mémoire (CD8, CD45RO), ainsi que par celle des cellules Th1, est associée à un meilleur pronostic dans le cancer colorectal (Galon et al., 2006). L'infiltration de la tumeur par les sous-types Th2 et Th17 est en revanche associée à un mauvais pronostic (Tosolini et al., 2011). La production d'IL17 par les cellules Th17 entraîne en effet la production locale de VEGF, favorisant ainsi l'angiogenèse et la croissance tumorale (Liu et al., 2011). Le rôle de l'infiltration des cellules Treg sur le pronostic est cependant assez controversé dans le cancer colorectal. Les cellules Treg pourrait empêcher la carcinogenèse colorectale en limitant le développement tumoral induit par l'inflammation (Mantovani et al., 2008). En revanche, si le cancer colorectal progresse, les cellules Treg pourrait alors inhiber la réponse anti-tumorale (Whiteside, 2012).

Les cellules NK jouent un rôle majeur dans la réponse immunitaire au cancer, par leur capacité à contrôler la croissance tumorale et la formation des métastases. Les NK ont deux types de récepteurs, les récepteurs activateurs tels que le NKG2D, et les récepteurs inhibiteurs tels que les KIR. Le récepteur NKG2D peut interagir avec différents ligands activateurs surexprimés par les cellules cancéreuses. Les KIR reconnaissent quant à eux le CMH-1, les cellules NK peuvent donc être activées par la diminution de l'expression du CMH-1 par les cellules cancéreuses (Carbone et al., 2005; Malmberg et al., 2008). Dans le CRC, une forte infiltration intra-tumorale de cellules NK est associée à un meilleur pronostic (Coca et al., 1997).

Les cellules NKT partagent les caractéristiques des cellules NK et des lymphocytes T. Ils peuvent reconnaitre des antigènes glycolipidiques présentés par le récepteur CD1d, un récepteur de la famille du CMH-1 (Robertson et al., 2014). L'activation des cellules NKT

entrainent une forte sécrétion de cytokines pro-inflammatoires et de molécules effectrices impliquées dans la mort cellulaire (perforine, Fas-L, TRAIL). Une augmentation de l'infiltration de la tumeur en cellules NKT est associée à un meilleur pronostic dans le cancer colorectal (Tachibana et al., 2005).

1.2. Les monocytes/macrophages

Les monocytes sont des cellules immunitaires avec des caractéristiques morphologiques propres, avec une forme irrégulière, un noyau ovale ou réniforme, et la présence de vésicules cytoplasmiques. Ceux-ci représentent environ 10% des cellules immunitaires circulantes dans le sang chez l'homme, et sont également présents dans la rate, Les monocytes peuvent rester dans la circulation jusqu'à deux jours. Ceux-ci sont ensuite éliminés s'ils ne sont pas recrutés dans un tissu.

Les monocytes proviennent de la moelle osseuse et sont issus de la différenciation des cellules souches hématopoïétiques (HSC) (Figure 14). Celles-ci prolifèrent et se différencient en passant par différents stades : le précurseur myéloïde commun (CMP), le précurseur des macrophages et des granulocytes (GMP), le précurseur commun des macrophages et des cellules dendritiques (MDP) puis finalement le précurseur des monocytes engagés (cMoP), un précurseur perdant l'expression du récepteur CD135 par rapport au précurseur MDP (Akashi et al., 2000; Fogg et al., 2006; Hettinger et al., 2013).

Le développement des monocytes est principalement régulé par le MCSF (ou CSF-1), produit par les cellules endothéliales, les cellules stromales, les fibroblastes et par les monocytes/macrophages eux même, dans le sang et les tissus. Il agit à travers le récepteur CSF-1R (Hamilton, 2008). Le GM-CSF est une autre cytokine permettant le développement des monocytes, celui-ci est cependant surexprimé uniquement en condition d'inflammation (Gasson, 1991).

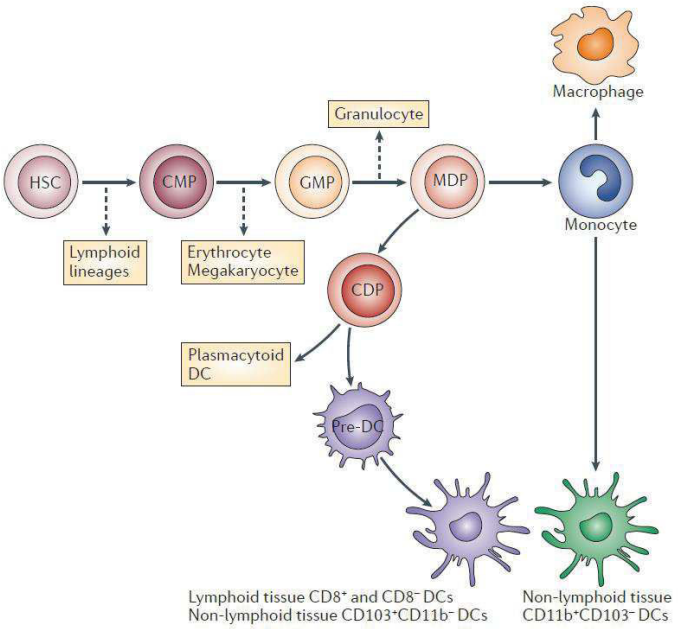

Figure 14 : Le système de différenciation des cellules phagocytaires mononucléaires (Chow et al., 2011)

Les monocytes circulant dans le sang sont hétérogènes. On peut en effet distinguer trois types de monocytes, basés sur l'expression des récepteurs CD14 (corécepteur du LPS) et CD16 (FcγRIII) à leur membrane (Ziegler-Heitbrock et al., 2010). Les monocytes dit classiques représentent 85% des monocytes et présentent une forte expression de CD14 mais pas d'expression de CD16. Les monocytes intermédiaires, soit environ 5% des monocytes, montrent une forte expression de CD14 et une faible expression de CD16. Les monocytes non classiques, (10% des monocytes) montrent quant à eux une forte expression de CD16 et une faible expression de CD14 (Ziegler-Heitbrock et al., 2010). Les monocytes classiques et intermédiaires ont des propriétés inflammatoires. Ceux-ci expriment fortement le récepteur aux chemokines CCR2, permettant leur réponse à la chemokine CCL2 et leur recrutement au niveau des sites inflammatoires (Tsou et al., 2007). Les monocytes non classiques patrouillent dans le sang et expriment fortement le récepteur aux chemokines CX3CR1, permettant leur réponse à la chemokine CX3CL1, une chemokine soluble et liée à la membrane des cellules endothéliales (Geissmann et al., 2003).

Les monocytes constituent un réservoir de précurseurs de cellules myéloïdes permettant le renouvellement des cellules dendritiques et des macrophages présents dans les tissus, ces derniers pouvant également se développer indépendamment (Boltjes and van Wijk, 2014; Liu et al., 2009). Les monocytes contribuent également à l'immunité innée par leurs capacités à

éliminer les microbes par phagocytose, la production de ROS, de NO et de cytokines pro-inflammatoires (Serbina et al., 2008).

Lorsque les monocytes sont recrutés dans un tissu, ceux-ci peuvent se différencier en macrophages. Les macrophages peuvent porter différents noms selon le tissu dans lequel ils sont situés (poumon..). Les macrophages sont des cellules plastiques pouvant être polarisés *in vitro* en deux phénotypes distincts (pro-inflammatoire M1 vs pro-tumoral M2) avec des rôles différents dans le cancer. Ces derniers sont les formes extrêmes de la polarisation, ils sont plutôt retrouvés sous différents états intermédiaires *in vivo*. Les macrophages intègrent en effet les différents signaux de leur microenvironnement et acquièrent différents phénotypes en fonction de ces signaux (Sica and Mantovani, 2012). La différenciation des monocytes en macrophages fait intervenir principalement les facteurs de transcription PU.1 et MafB (Kelly et al., 2000; Scott et al., 1994). La polarisation des macrophages est ensuite déterminée suite à l'activation de différents facteurs conduisant soit vers un phénotype M1 (STAT1, STAT5,…) ou M2 (STAT6, PPARγ,…) en réponse à l'environnement (Figure 15).

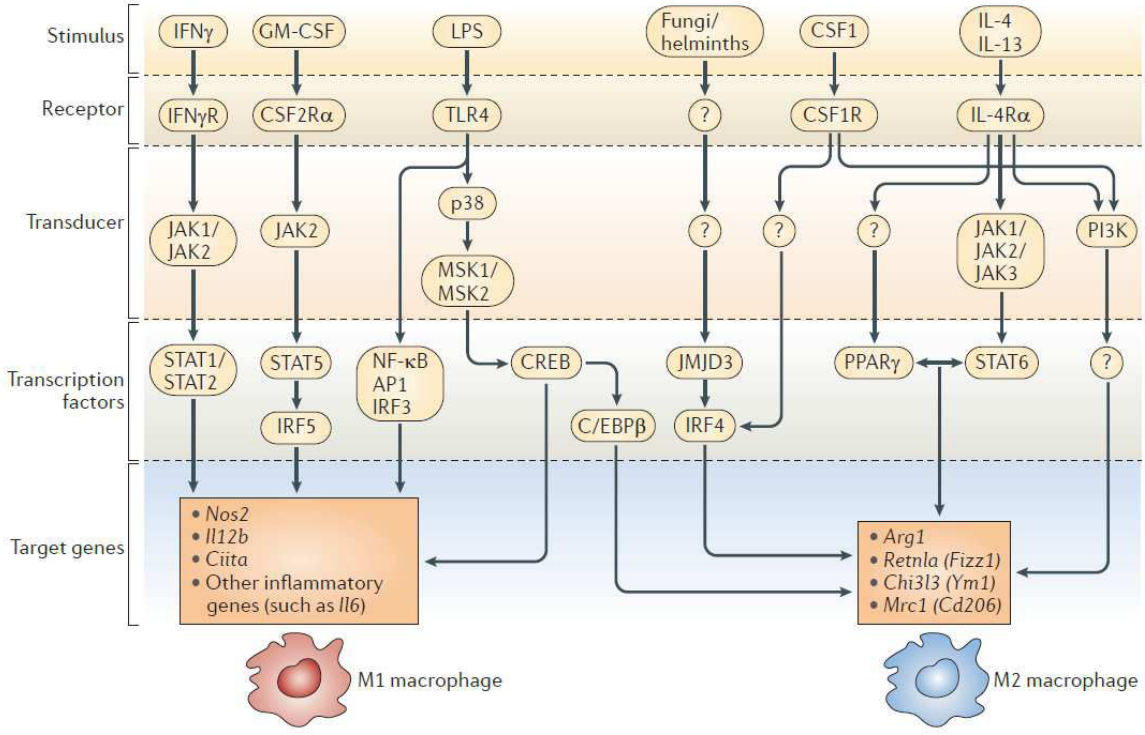

Figure 15 : Les voies de signalisation intervenant dans la polarisation des macrophages
(Lawrence and Natoli, 2011)

Les macrophages M1 sont induits en présence de cytokines pro-inflammatoires telles que le GM-CSF et l'IFNγ, ou de composés microbiens comme le LPS (Figure 16). Ces macrophages peuvent phagocyter les cellules tumorales et les bactéries liées à des anticorps grâce à leur expression de récepteurs aux fragments constants des immunoglobulines tels que le CD64 (antibody-dependent cell-mediated cytotoxicity, ADCC). Ils agissent également comme des CPA, par l'expression du CMH-II et de molécules co-stimulatrices telles que CD86 et CD80 (Ambarus et al., 2012). Ces macrophages sont caractérisés par la production de molécules effectrices telles que les ROS et de cytokines pro-inflammatoires (Il-1β, TNFα, IL-6, IL-12, IL-23) (Sica and Mantovani, 2012). La production de chemokines telles que CXCL9 et CXCL10 leur permet d'attirer les lymphocytes Th1.

Les macrophages peuvent également être polarisés vers un phénotype M2, en présence de MCSF, d'Il-4 et d'Il-13 (Gordon and Taylor, 2005). Les macrophages M2 présentent une forte production d'Il-10, ainsi qu'une faible capacité à présenter les antigènes (Noel et al., 2004). Les macrophages M2 peuvent produire les chemokines CCL17, CCL22 et CCL24 afin de recruter les cellules Treg, Th2, éosinophiles et basophiles (Mantovani et al., 2002; Martinez et al., 2006). Ces macrophages participent à la résolution de l'inflammation, contribuent à la protection contre les parasites, et peuvent promouvoir la réparation des blessures, l'angiogenèse et le remodelage des tissus (Biswas and Mantovani, 2010).

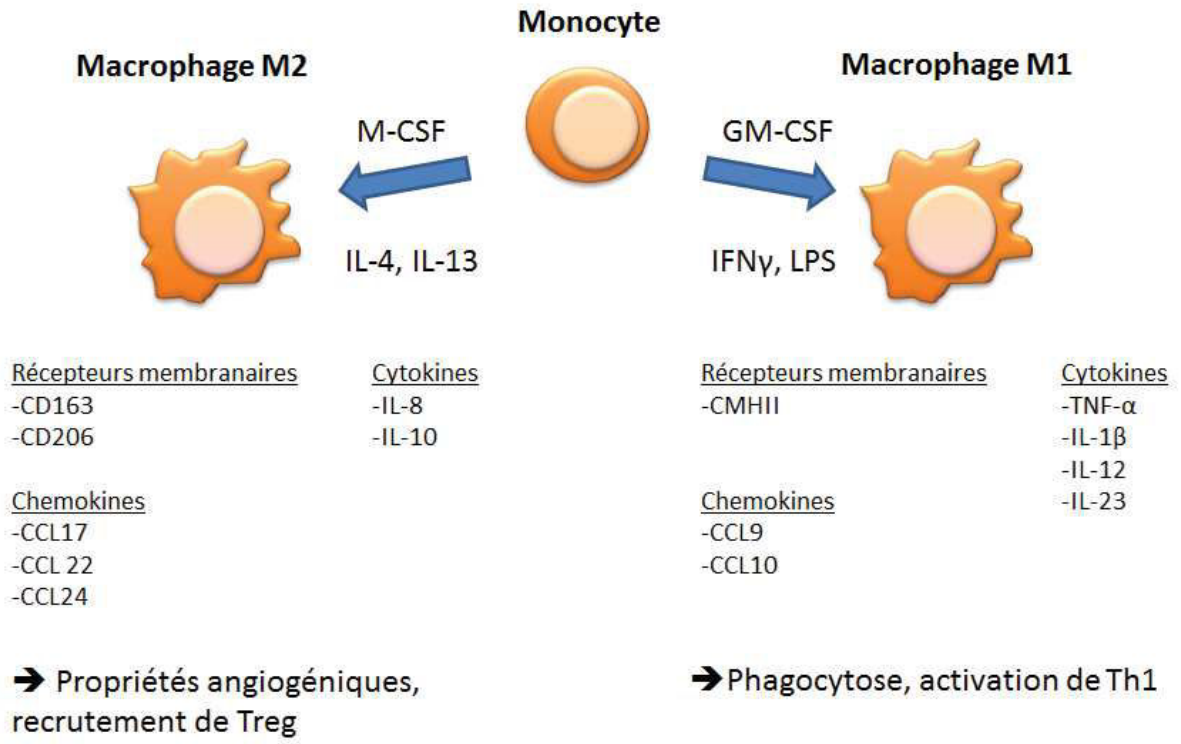

Figure 16 : Caractéristiques des deux types de macrophages

Les macrophages qui infiltrent les tumeurs et favorisent leur développement sont généralement proches du phénotype M2. Ils participent au remodelage de la matrice extracellulaire par la production de protéines protéolytiques, telles que la plasmine, la cathepsine B et les MMP (Gocheva et al., 2010; Nagakawa et al., 2002). Ils sont ainsi associés au pouvoir métastatique de différentes tumeurs (Lin et al., 2011; Qing et al., 2012). Ils favorisent l'angiogenèse par la production de différentes molécules comme le TGF-β, le VEGF et le PDGF (Granata et al., 2010; Murdoch et al., 2008; Schoppmann et al., 2002). Les macrophages associés aux tumeurs exercent également une activité immunosuppressive par l'expression de différentes molécules telles que PD-L1, le TGF-β, l'arginase-1, IDO, et l'Il-10 (Mantovani and Sica, 2010).

L'infiltration de macrophages est majoritairement un facteur de mauvais pronostic dans différents cancers, notamment les cancers gastriques, ovariens, mammaires et de la vessie, et sont associés à la progression tumorale et au développement de métastases. Une polarisation des macrophages vers un phénotype M2 semblent donc être prédominante dans ces cancers (Zhang et al., 2012). Le blocage du CSF-R1 des macrophages dans différents cancers permet

de limiter leur polarisation vers un phénotype M2, conduisant ainsi au recrutement de lymphocytes T et à la régression tumorale (Pyonteck et al., 2013; Ries et al., 2014).

L'infiltration des macrophages est en revanche associée à un bon pronostic dans le cancer colorectal (Chaput et al., 2013; Forssell et al., 2007). Les cancers colorectaux de type MSI sont associés à une plus forte infiltration de macrophages au niveau du front tumoral (De Smedt et al., 2015). Les macrophages infiltrant les cancers colorectaux ont un profil mixte M1/M2 et sont capables d'inhiber la croissance du cancer colorectal et d'initier une réponse anti-tumorale suite à l'activation des lymphocytes T (Engstrom et al., 2014; Ong et al., 2012). Les fonctions des macrophages M1 semblent donc prédominer dans ces cancers (Edin et al., 2012). Les mécanismes liés à cette polarisation des macrophages dans les cancers colorectaux ne sont pas connus, mais pourraient être liés à l'environnement particulier de ce cancer, comme par exemple la stimulation des macrophages par le microbiote intestinal. Les cellules cancéreuses colorectales sont également capable de sécréter des facteurs pouvant polariser les macrophages vers un phénotype mixte M1/M2, mais ceux-ci ne sont pas encore clairement identifiés (Caras et al., 2011; Edin et al., 2013; Wu et al., 2014). La production de GM-CSF par les cellules cancéreuses colorectales est, par exemple, associée à un bon pronostic (Nebiker et al., 2014).

1.3. Les antigènes associés aux tumeurs dans le cancer colorectal

Les antigènes associés aux tumeurs permettent une réponse immunitaire médiée par l'immunité cellulaire et humorale. Plusieurs types d'antigènes sont exprimés par la tumeur. Dans le cancer colorectal, les antigènes de tumeur les plus fréquents sont des antigènes normaux exprimés à un faible niveau dans les cellules normales et les tissus embryonnaires et à un haut niveau dans les cellules tumorales. Le plus connu d'entre eux est l'antigène carcino-embryonnaire (CEA), qui est exprimé normalement dans les tissus fœtaux, et surexprimé dans le CRC (Hammarstrom, 1999). Les protéines Ep-Cam, HER-2/neu (Nagorsen et al., 2000), MUC-1 et p56, peuvent également servir d'antigènes de tumeur. Des réponses immunitaires peuvent également être dirigées contre des néo-antigènes, générés après mutation dans la séquence des protéines. Des mutations dans p53 et k-Ras peuvent ainsi activer le système immunitaire (Keogh et al., 2001).

Les cancers colorectaux de type MSI sont associés à une forte densité de lymphocytes infiltrant les tumeurs et ont un meilleur pronostic que les CRC microsatellite stable (Benatti et al., 2005; Smyrk et al., 2001). L'instabilité microsatellitaire dans ces cancers entraîne des mutations dans le cadre de lecture de gènes cibles conduisant non seulement à l'inactivation de ces gènes, mais aussi à l'apparition de nouveaux antigènes immunogéniques. Des mutations dans les gènes du TGFβR2 (Saeterdal et al., 2001), de l'OGT (Ripberger et al., 2003), de MSH3 (Garbe et al., 2011), de la caspase 5, d'ASTE1 et de PTEN sont notamment capables d' induire une réponse immunitaire T spécifique. On retrouve ainsi 10 à 50 fois plus de néo-antigènes dans les cancers colorectaux MSI que dans les tumeurs MSS (Llosa et al 2015). Le nombre de mutations corrèle avec la densité des lymphocytes T infiltrant la tumeur et la progression tumorale (Maby et al., 2015; Tougeron et al., 2009).

2. Les mécanismes d'échappement du cancer colorectal au système immunitaire

2.1. Induction de lymphocytes T régulateurs (Treg)

L'induction de cellules immunosuppressives est le mécanisme majeur d'échappement au système immunitaire de l'hôte. Les cellules Treg peuvent bloquer la réponse immunitaire contre les tumeurs par la sécrétion de cytokines telles que l'IL-10 et le TGF-β, ainsi que par la sécrétion de métabolites immunosuppressives telles que l'adénosine. Elles peuvent également inhiber les lymphocytes T CD8+ par des mécanismes dépendants des contacts entre cellules.

L'accumulation de cellules Treg dans les tumeurs peut s'expliquer par plusieurs mécanismes (Tanchot et al., 2013). Le premier mécanisme repose sur la conversion des lymphocytes T CD4+ en cellules Treg en réponse à différents signaux comme le TGF-β. Les tumeurs peuvent également recruter préférentiellement les cellules Treg à travers la production de chemokines telles que CCL17, CCL22 et CCL28 (Facciabene et al., 2011; Pere et al., 2011). Le VEGF-A sécrété par les tumeurs semble également jouer un rôle important dans l'induction des cellules Treg. Celui-ci peut en effet inhiber la maturation des cellules dendritiques. Les cellules dendritiques immatures, sécrétant du TGF-β, peuvent alors favoriser la conversion les lymphocytes T CD4+ en Treg (Belkaid and Oldenhove, 2008; Ghiringhelli et al., 2005). Le VEGF-A peut également promouvoir l'expansion des cellules Treg à travers leur récepteur VEGFR-2 (Terme et al., 2013).

2.2. Diminution de l'expression du CMH-1

Le CMH-1 est retrouvé sous-exprimé dans plus de 70% des cancers colorectaux (Menon et al., 2002). La perte complète du CMH-1 est rare et est principalement due à l'inactivation du gène de la β2-microglobuline dans les tumeurs MSI ou à la diminution de l'expression des protéines LMP7 et TAP2, impliquées dans la formation et le transport du peptide à présenter, dans les tumeurs MSS (Cabrera et al., 2003). La diminution de l'expression peut être due à la perte allélique du gène *HLA*, codant pour le CMH-1, suite à une non-disjonction du chromosome ou à une recombinaison mitotique, ou à la perte de son expression. Les cellules cancéreuses peuvent également présenter un CMH-1 non fonctionnel suite à des mutations ponctuelles ou des délétions.

La diminution de l'expression du CMH-1 est associée à un mauvais pronostic en comparaison avec celui des tumeurs l'exprimant fortement. En revanche, les tumeurs avec une perte complète de son expression montrent le même pronostic que celles avec une forte expression, ce qui pourrait être dû à une forte activité des cellules NK dans ces tumeurs (Watson et al., 2006).

2.3. La surexpression de PD-L1

En raison de la forte activité du système immunitaire, et notamment des lymphocytes T CD8+ et Th1 dans les cancers colorectaux MSI, ceux-ci ne devraient normalement jamais se développer ou régresser. La progression tumorale peut s'expliquer par la surexpression par les lymphocytes T issus de ces tumeurs de différentes protéines impliquées dans les points de contrôle immunitaires, comparativement aux cancers MSS. On retrouve parmi ceux-ci PD-1 et CTLA-4 (Gubin et al., 2014).

Au cours d'une infection, l'expression de PD-1 par les lymphocytes T est augmentée, puis diminue lorsque l'antigène n'est plus présent. En revanche, lorsque l'antigène persiste, comme dans le cancer, l'expression de PD-1 par les lymphocytes T se maintient, entraînant une diminution de leurs fonctions effectrices et de leurs capacités prolifératives (épuisement des lymphocytes T). L'interaction de PD-1 avec son ligand, PD-L1, entraîne l'inhibition des fonctions immunitaires des lymphocytes T, telles que la production de cytokines pro-

inflammatoires (IFNγ, IL-2), la motilité, la capacité à interagir avec les cellules dendritiques et les cellules à éliminer. L'expression de PD-L1 par les cellules cancéreuses est retrouvée dans différents cancers tels que les mélanomes (Taube et al., 2012). Celui-ci est en revanche faiblement exprimé par les cellules cancéreuses dans les cancers colorectaux, mais est cependant retrouvé sur les cellules myéloïdes et contribuerait à l'inhibition de l'activation des lymphocytes T (Llosa et al., 2015). Des anticorps dirigés contre PD-1 et PD-L1 ont montré une forte efficacité chez les patients atteints de mélanomes, de cancers rénaux et pulmonaires. Des essais cliniques ont été débutés afin d'évaluer l'effet du blocage de PD-1 chez les patients atteints de cancers colorectaux MSI (Le et al., 2015).

CTLA-4 est un récepteur situé à la membrane des lymphocytes T et impliqué dans l'inhibition de leur activité en interagissant avec les molécules de co-stimulation CD80 et CD86 présentées par les CPA. Des anticorps dirigés contre CTLA-4 entraînent une augmentation de l'activation des lymphocytes T dans les mélanomes métastatiques (Buchbinder and Hodi, 2015).

3. Interactions d'HSP110 avec le système immunitaire

3.1. Développement de vaccins contre le cancer

Plusieurs équipes se sont intéressées à l'utilisation des propriétés chaperons des HSP pour la réalisation de vaccins dirigés contre les cancers. En effet, dans les cancers, les HSP sont surexprimées et permettent, entre autres, par leur activité chaperon, d'éviter l'agrégation des protéines oncogéniques mutées essentielles à la tumorigenèse. Les HSP issues de ces tumeurs sont donc censées porter leurs antigènes et être capables d'induire une réponse immunitaire anti-tumorale spécifique (Ishii et al., 1999; Srivastava et al., 1986; Udono and Srivastava, 1993).

La forte capacité de stabilisation d'HSP110 a été utilisée afin de réaliser plusieurs vaccins. Des animaux immunisés avec de l'HSP110 purifiée à partir de différentes lignées tumorales (colon, mélanome,…) de souris développent une forte réponse immunitaire anti-tumorale (Wang et al., 2001). Ainsi, des complexes comprenant HSP110 et le domaine intracellulaire d'HER2/neu entraînent une réponse des lymphocytes T CD4+ et CD8+ spécifique. Ce vaccin est capable de ralentir ou inhiber le développement tumoral mammaire (Manjili et al., 2002; Manjili et al., 2003). Des résultats similaires sont obtenus lorsque des

souris, dans lesquelles ont été injectées des cellules B16 (mélanome de souris), sont immunisées avec de l'HSP110 complexée avec la protéine gp100, un antigène tumoral associé au mélanome (Wang et al., 2003). L'HSP110 complexée avec l'anhydrase carbonique IX, un antigène de carcinome de cellules rénales, génère quant à elle une puissante réponse anti-tumorale contre les carcinomes rénaux de souris (Kim et al., 2007). Seul le domaine de liaison au peptide d'HSP110 est essentiel pour son rôle dans la vaccination (Park et al., 2006).

La promotion de l'activation immunitaire par les HSP dépend de leur capacité à transporter et délivrer les antigènes aux CPA pour leur présentation croisée (Murshid et al., 2008). La capture des complexes HSP-Antigènes par les CPA est réalisée par endocytose grâce à différents récepteurs, tels que CD91 et LOX-1 (Binder et al., 2000; Delneste et al., 2002).

La production d'un vaccin ne nécessite pas d'extrait de la tumeur, cette approche pourrait être utilisée comme adjuvant pour les patients atteints d'un cancer, mais également à titre préventif chez les patients ayant un fort risque de récurrence du cancer.

3.2. Interaction entre HSP110 et les récepteurs scavenger

Les récepteurs scavenger tels que SR-A et SREC-1 sont exprimés par les cellules dendritiques et par les macrophages. Les récepteurs Scavenger peuvent interagir avec de nombreux ligands tels que les lipoprotéines oxydées, les motifs associés aux pathogènes (PAMP) et les cellules apoptotiques (Canton et al., 2013).

L'HSP110 présente dans le milieu extracellulaire peut interagir avec ces récepteurs (Facciponte et al., 2007). Cette interaction aurait un effet tolérogène sur le système immunitaire. En effet, la vaccination de souris déficientes en SR-A par des complexes HSP110-gp100 entraîne une plus forte réponse anti-tumorale (Qian et al., 2011; Wang et al., 2007). Cette déplétion permet également la reconnaissance par le système immunitaire de plusieurs tumeurs faiblement immunogéniques (Wang et al., 2007). A l'inverse, l'induction d'une hépatite par la concanavaline A dans ces souris entraîne une sur-activation des lymphocytes T et une augmentation de la mortalité (Zuo et al., 2013). La présence de SR-A semble donc réduire l'activité immunostimulatrice des CPA.

SR-A agit en réduisant l'expression des molécules de co-stimulation et la production de cytokines pro-inflammatoires, ce qui réduit la présentation des antigènes.et diminue la réponse

des lymphocytes T (Yi et al., 2011). SR-A pourrait supprimer l'activation de la voie NF-κB suite à la liaison d'un ligand sur le récepteur TLR4, en interférant avec la trimerisation et l'ubiquitination du récepteur TRAF6 (Ohnishi et al., 2011). L'activation de la voie NF-κB est essentielle pour l'activation des CPA.

3.3. Induction de l'expression de CD1d par HSP110 extracellulaire

Le récepteur CD1d appartient à la famille des récepteurs de CD1 et présente une structure et une fonction proche de ceux du CMH de classe I et II (Blumberg et al., 1995; Briken et al., 2000). On le retrouve aussi bien à la surface des monocytes et des cellules dendritiques que de différents types de cellules épithéliales dont les cellules épithéliales intestinales (Blumberg et al., 1991; Canchis et al., 1993). La présentation d'antigènes glycolipidiques par le récepteur CD1d des CPA aux cellules NKT permet le déclenchement d'une réaction inflammatoire (Briken et al., 2000).

La fonction du récepteur CD1d sur les cellules épithéliales a principalement été étudiée dans des modèles d'inflammation de l'intestin, où il est retrouvé sous-exprimé (Heller et al., 2002; Saubermann et al., 2000). L'activation de celui-ci entraîne la production d'IL-10 à travers la voie STAT3. L'IL-10, à travers différentes voies autocrines, peut protéger l'épithélium des effets délétères des cytokines pro-inflammatoires comme l'IFNγ (Colgan et al., 1999).

Contrairement aux tissus fraichement isolés, les lignées cellulaires dérivées de l'épithélium exprime faiblement le récepteur CD1d. Cependant, lorsque ces cellules sont stimulées par des composants extracellulaires du milieu intestinal, ceux-ci entraînent une augmentation de l'expression du récepteur par les cellules épithéliales intestinales. L'analyse de ce milieu a permis d'identifier HSP110 comme le facteur clef impliqué dans cette induction de l'expression de CD1d, de manière indépendante du LPS (Colgan et al., 2003).

L'HSP110 extracellulaire est ainsi capable d'induire l'expression de CD1d, de l'IL-10 et sa propre expression par les cellules épithéliales intestinales par activation de la voie STAT3, constituant ainsi une boucle de régulation de l'homéostasie de la muqueuse intestinale (Figure 17). Le mécanisme de sécrétion d'HSP110, ni son récepteur n'ont encore été identifiés (Olszak et al., 2014).

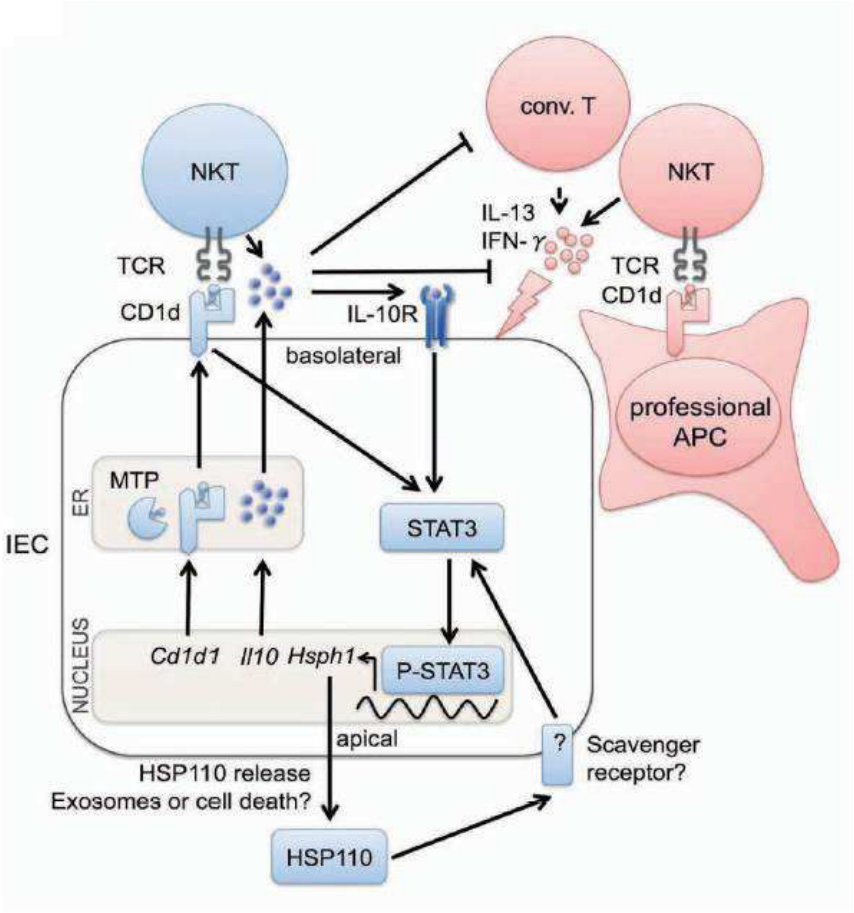

Figure 17 : Modèle proposé pour la signalisation de CD1d dans l'épithélium intestinal
Les effets protecteurs (en bleu) et délétères (en rouge) de la présentation d'antigènes glycolipidiques dans l'inflammation intestinale. Le recrutement de CPA contribue à l'inflammation à travers leur récepteur CD1d et l'activation des NKT. En revanche, l'activation du CD1d des cellules épithéliales intestinales montre des fonctions protectives par l'activation de la transcription de *Cd1d1, IL10 et Hsph1* de façon STAT3 dépendante. La sécrétion d'IL-10 et d' HSP110 renforce cet effet protectif de manière STAT3 dépendante (Olszak et al., 2014)

Résultats

Article 1: Mutation of *HSP110* Inhibits Tumor Growth Through a NMD Druggable Pathway in Colorectal Cancer

Notre équipe, en collaboration avec l'équipe du Dr Duval, (INSERM U938) a mis en évidence l'expression d'un mutant d'HSP110, nommé HSP110ΔE9, dans les cancers colorectaux de type microsatellite instable. HSP110ΔE9 agit comme un dominant négatif d'HSP110, en se liant et en inhibant ses fonctions chaperons et anti-apoptotiques. De façon intéressante, l'expression de ce mutant est associée à un bon pronostic pour les patients et à une meilleure réponse à la chimiothérapie.

HSP110 est impliquée dans la régulation de l'activité du facteur de transcription STAT3 en condition de choc thermique dans des lignées de cellules non cancéreuses. (Saito et al., 2014; Yamagishi et al., 2009). Dans une première partie de mon travail, je me suis intéressé au rôle potentiel que pourrait jouer la surexpression d'HSP110 dans le cancer colorectal, et notamment sur la voie de signalisation oncogénique STAT3. L'effet de l'inhibition des fonctions d'HSP110 a également été déterminé à travers la surexpression d'HSP110ΔE9.

Mutation of *HSP110* Inhibits Tumor Growth Through a NMD Druggable Pathway in Colorectal Cancer

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ABSTRACT

We recently reported the first mutation of a chaperone in cancer thus far 1. It affects HSP110 in the subset of colorectal cancer (CRC) displaying microsatellite instability (MSI) 2-4. The HSP110 mutation is frameshift and leads to the synthesis of a truncated, dominant negative mutant HSP110 isoform by exon 9 skipping (HSP110DE9) that sensitizes tumor cells to chemotherapeutic agents 1,5. Here we show that the HSP110 mutation also results in strong anticancer effects, e.g. by down-regulation of STAT3 signaling in MSI colon tumors. The forced overexpression of HSP110DE9 mutant or silencing of HSP110 expression was found to strongly inhibit both MSI and non-MSI colon tumor growth in xenograft mouse model. However, in MSI cancer cell, expression of endogenous HSP110DE9 transcript was censored by nonsense-mediated mRNA decay (NMD), like other MSI-driven frameshift mutant mRNAs. NMD blockade with the drug amlexanox, used in the clinic, allowed significant re-expression of frameshift mutant mRNAs including HSP110DE9 in MSI tumor cells and mutant dystrophin transcript in a dedicated transgenic mouse model. Systemic administration of this drug led to strong inhibition of the growth of MSI but no MSS colon tumor xenografts. Our findings highlight HSP110 as a master gene whose inhibition abrogates colon tumor development and NMD blockade as a seducing strategy allowing the re-expression of MSI-driven anticancer mutants such as the HSP110DE9 for personalized medicine of MSI CRC patients.

Over the past twenty years, many studies on colorectal cancer (CRC) displaying MSI due to mismatch repair (MMR) deficiency have reported frameshift truncating mutations in coding DNA repeats located within genes involved in various cancer-related pathways (for review, see 6,7). With others (e.g. BRAF activating mutations), these MSI-driven mutations are thought to include the essential events promoting MSI colon tumor development. Besides, our group more recently reported somatic deletions within a T17 intronic microsatellite at the junction between intron 8 and exon 9 of the HSP110 chaperone gene in human tumors (HT17) 1. Strikingly, the frequent HSP110 mutation was shown to have deleterious effect on HSP110 activity with regards to apoptosis and chemotherapy resistance in vitro, causing MSI tumor cells to become sensitized to several chemotherapeutic agents. In primary MSI CRCs, we observed that a decrease in the length of HT17 correlated with an increased HSP110DE9/HSP110 mRNA ratio and progressive inhibition of HSP110 activity in tumor cells. In line with this, about 25% of patients with stages II–III MSI colorectal tumors were shown to have an excellent response to chemotherapy, due to large, biallelic deletions in HT17 in tumor DNA 5,8.

An important question still to be addressed is whether HSP110 mutation also has a detrimental pathophysiological role during MSI CRC development. This is especially relevant given increasing evidence for the role of heat shock proteins in colon cancer and the recently reported physiological role of HSP110 in colonic mucosa 9. Here we observed a marked decrease in tumor growth in xenografts derived from HCT116 (MSI) sub-clones with large (no remaining HSP110 wild type activity) vs small (positive remaining HSP110 wild type activity) T17 deletion (see 5 for further details

concerning the analyzed sub-clones) (Fig. 1A). Moreover, stable overexpression of HSP110DE9 also led to drastic decreased tumor growth in xenografted nude mice in the HCT116 MSI colorectal tumor model compared to HSP110wt or empty vector (Fig. 1B and Supplementary Figure S1A). Similar results were obtained in MSS (no HSP110 mutation) colorectal (SW480) and MSS gastric (TMK1) tumor xenograft models, demonstrating the anticancer impact of HPS110DE9 following its forced overexpression in both MSI and MSS gastrointestinal tumor models. Finally, these anticancer effects were reproducibly observed when treating HCT116 or SW480 CRC cells with ShRNA targeted to HSP110 (Fig. 1C and Supplementary Figure S1B). We concluded that, besides its previously reported chemosensitizing impact in MSI colon cancer 1,5, mutation of HT17 had drastic anticancer effects by leading to strong inhibition of MSI tumor growth. As a candidate mechanism among putative others, we demonstrated the modulation of STAT3 activity by HSP110 in colon tumor cells, as reported recently 9. Briefly, HSP110 expression was first confirmed to have a positive impact on cell proliferation and STAT3 activity in our CRC models (see further details in Supplementary Fig. S2A). The level of phosphorylated STAT3 was shown to be significantly higher in the HCT116 sub-clone with a small vs large T17 deletion (Fig. 1D, left panel). Moreover, in both SW480 (MSS) and HCT116 (MSI) cells, the HSP110DE9 dominant negative mutant inhibited STAT3 phosphorylation induced by HSP110 in a dose-dependent manner (Fig. 1D, middle panel). Consequently, HSP110DE9 also blocked the expression of genes downstream of STAT3 such as c-MYC, MCL1, CCND1 and BCL-XL (Fig. 1D, right panel). Similar to HSP110DE9, the STAT3 inhibitor AG490 provoked a dose-dependent reduction in the accumulation of S-phase cells induced by HSP110 and in the ability of HSP110 to induce STAT3 downstream genes such as c-MYC and CCND1 (Supplementary Figure S2). Finally, modulation of STAT3 activity was found to be dependent on the mutation status of HSP110 T17 in both MSI tumor xenografts and primary colon cancers (Fig. 1E). Thus, the strong inhibition of MSI tumor growth we observed due to HSP110 mutation in MSI CRC was due, at least in part, to the down regulation of STAT3 signaling 9-11 but we assume it should be also related to other HSP110 dependent processes.

Although the above findings report a deleterious impact of HSP110 mutation in MSI colon tumors, the nonsense-mediated mRNA decay (NMD) system is responsible for rapid degradation of mutant mRNAs containing a premature termination codon (PTC) such as the one encoding the HSP110DE9 mutant protein 12. Therefore, we aimed at evaluating the impact of NMD activity on the expression of endogenous HSP110DE9 mutant mRNA in MSI CRC cell lines and primary tumors. NMD is mediated through the assembly of protein complexes that include members of the UPF family. UPF1 and UPF2 play a central role in NMD 13,14. In MSI colon tumors, we previously demonstrated that the efficiency of NMD for the degradation of mutant mRNAs was highly variable in MSI CRC tumors and dependent on the MSI target genes considered 15. Here we showed that, similar to other PTC-containing transcripts (e.g. TGFBR2, MSH3), treatment with the translation inhibitor cycloheximide (CHX) led to increased expression of HSP110DE9 mRNA in MSI CRC cell lines (Fig. 2A). Inhibition of HSP110DE9 mRNA expression and other mutated PTC-containing transcripts (TGFBR2, MSH3) by NMD was further demonstrated by using siRNA and shRNA targeted to NMD factor UPF1 in HCT116 (Fig. 2B). Concordantly, although MSI-

induced deletions in the HSP110 T17 repeat led to decreased expression of wild type HSP110 mRNA in primary tumors, we did not detect a concomitant increase of the HSP110DE9 transcript (Fig. 2C, right panel). Similar results were observed in CRC cell lines, although a weak increase of the HSP110DE9 transcript was observed in MSI CRC cells displaying large vs small T17 deletions (Fig. 2C, left panel. See also Fig. 2D and Supplementary Figure S3A the quantification study of all HSP110 mRNAs we performed in a large series of primary colon tumors, adenomas and paired normal mucosa). We concluded that the expression of HSP110DE9 mRNA was efficiently censored by NMD in MSI colon tumors, indicating that the aberrant HSP110DE9 protein does not significantly impact on MSI tumorigenesis by itself and the inhibition of HSP110 activity following somatic deletion of HT17 in MSI colon tumors is mainly due to the concomitant loss of wild type HSP110 expression (see the mechanistic model we propose in Fig. 2E).

The above reported findings and recent others suggest that inhibition of HSP110 could be an important factor in colon cancer therapy. Unfortunately, no inhibitors of HSP110 are currently available and the dominant negative HSP110DE9 truncated protein is too big and with important drawbacks to be used in the clinical setting. Although it has been shown that NMD targeted HSP110DE9 but also many other PTC-containing mutant mRNAs in MSI colon cancer with yet unknown functional consequences if any, we hypothesized that the inhibition of its overall activity could be of therapeutic interest in the MSI tumor context. In line with this hypothesis, we first observed that prolonged inhibition of UPF1 or UPF2 expression using shRNA led to decreased CRC cell proliferation (Fig. 3A, left panel) and significant decreased of MSI tumor xenografts (Fig. 3A, right panel). Concordantly, the endogenous expression of UPF1 (Fig. 3B, left panel) and other NMD factors (Fig. 3B, right panel) were higher in MSI compared to MSS primary CRC, indicating a putative oncogenic role for this system in MSI tumorigenesis. Using a firefly/renilla NMD reporter system (Fig. 3C upper left panel; Boelz, BBRC, 2006), Amlexanox, a known inhibitor of NMD 16, was shown to efficiently inhibit NMD in our CRC models in a dose dependent manner (HCT116, SW480; Fig. 3C, upper right panel) and to induce the re-expression of aberrant mRNAs such as MSH3 and HSP110DE9 (Fig. 3C, lower panel). Very interestingly, treatment with this agent led to the decrease of cell proliferation in HCT116 (MSI) but not in SW480 (MSS) tumor cells (Fig. 3D). Amlexanox has been in clinical use for decades for the treatment of recurrent aphthous stomatitis and we thus try to inhibit NMD by systemic administration in mouse. Amlexanox was shown to inhibit NMD in a dystrophin transgenic mouse model, as shown by the significantly higher amount of dystrophin PTC-mRNA in animals exposed to the drug compared to those exposed to DMSO (Fig. 4A). Treatment with this agent led to significant inhibition of tumor growth in mice xenografted with HCT116 cells compared to untreated animals receiving mock buffer (Fig. 4B, middle left panel). In contrast, Amlexanox had no effect on the growth of SW480 xenografts under the same experimental conditions (Fig. 4B, middle right panel). We concluded that inhibition of NMD could be of interest for specific treatment of MSI CRC patients. Amlexanox was reported to inhibit other proteins including the protein kinases TBK1 and IKK-ε in obese mice improving metabolic parameters 17. Besides, we assume that this NMD inhibitor significantly impact the expression of dozens of additional MSI-driven target

mutants in MSI CRC cells, so that the part of tumor growth inhibition that could be attributed to HSP110DE9 stabilization itself is difficult to predict. This will have to be further evaluated in future studies. Nevertheless, by xenografting mice with HCT116 sub-clones displaying large or small T17 deletion, we observed significant impact of Amlexanox treatment on tumor growth in both contexts, suggesting that this agent could be of clinically relevant in the great majority of MSI CRC patients (Fig. 4B, lower panel). It is noteworthy that there was no evidence of toxicity from amlexanox in our animal experiments, in line with clinical experience for the systemic treatment of aphthous ulcers in human 18.

In this study, we report the drastic anticancer effect of the HSP110 mutation in colorectal cancer. Our data also show how the deleterious impact of this alteration is attenuated due to active NMD in MSI tumor cells (See our model Fig. 4C). We also report for the first time the opportunity to inhibit NMD by the use of amlexanox that warrants testing in clinical trials as a novel anticancer agent for the personalized treatment of MSI cancer patients.

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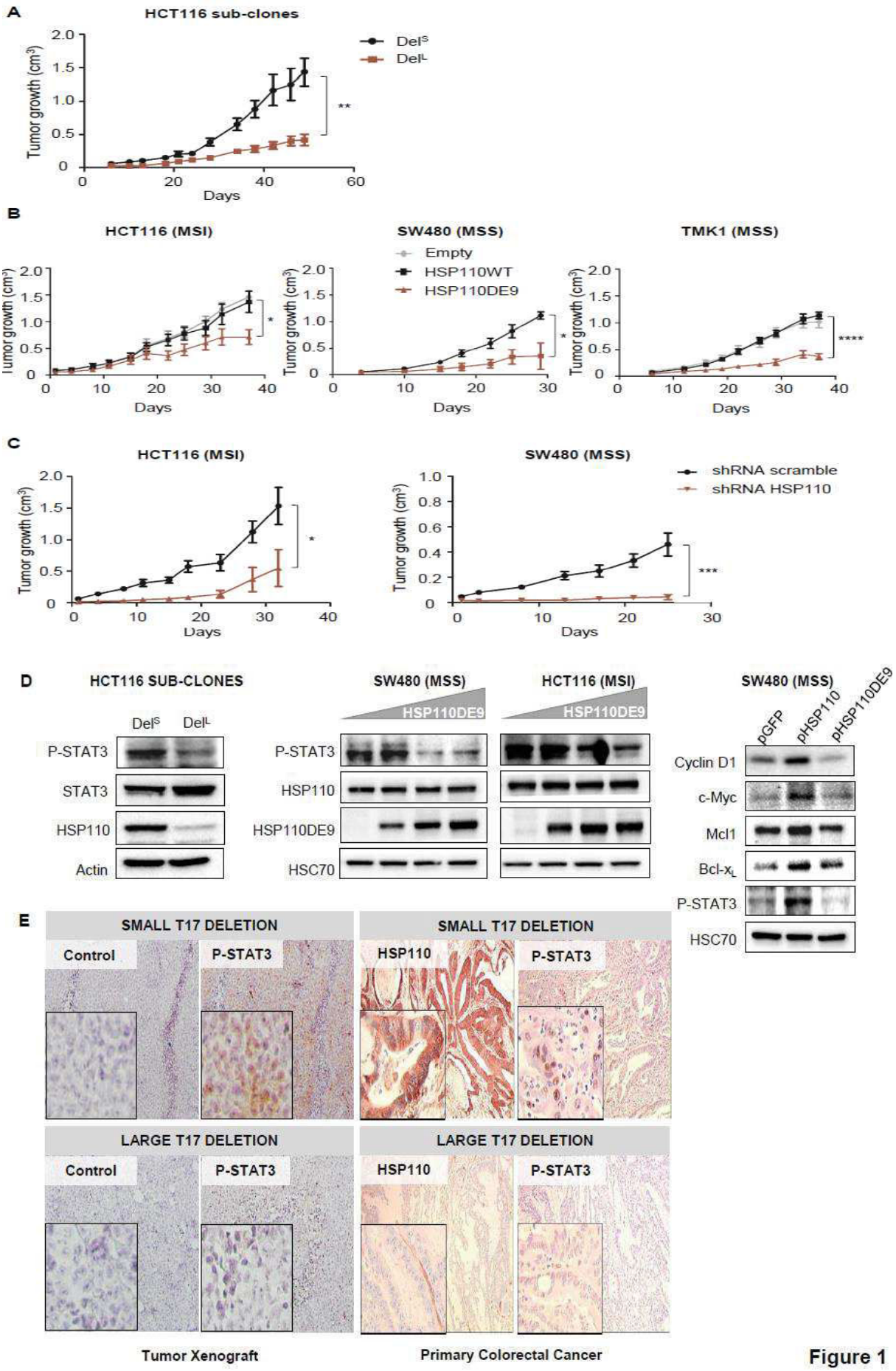

A
HCT116 sub-clones
Tumor growth (cm3)
Days
DelS
DelL
**
B
HCT116 (MSI)
SW480 (MSS)
TMK1 (MSS)
Empty
HSP110WT
HSP110DE9
*
****
C
HCT116 (MSI)
SW480 (MSS)
shRNA scramble
shRNA HSP110
***
D
HCT116 SUB-CLONES
SW480 (MSS)
HCT116 (MSI)
HSP110DE9
P-STAT3
STAT3
HSP110
Actin
HSP110DE9
HSC70
pGFP
pHSP110
pHSP110DE9
Cyclin D1
c-Myc
Mcl1
Bcl-xL
E
SMALL T17 DELETION
LARGE T17 DELETION
Control
P-STAT3
HSP110
Tumor Xenograft
Primary Colorectal Cancer

Figure 1

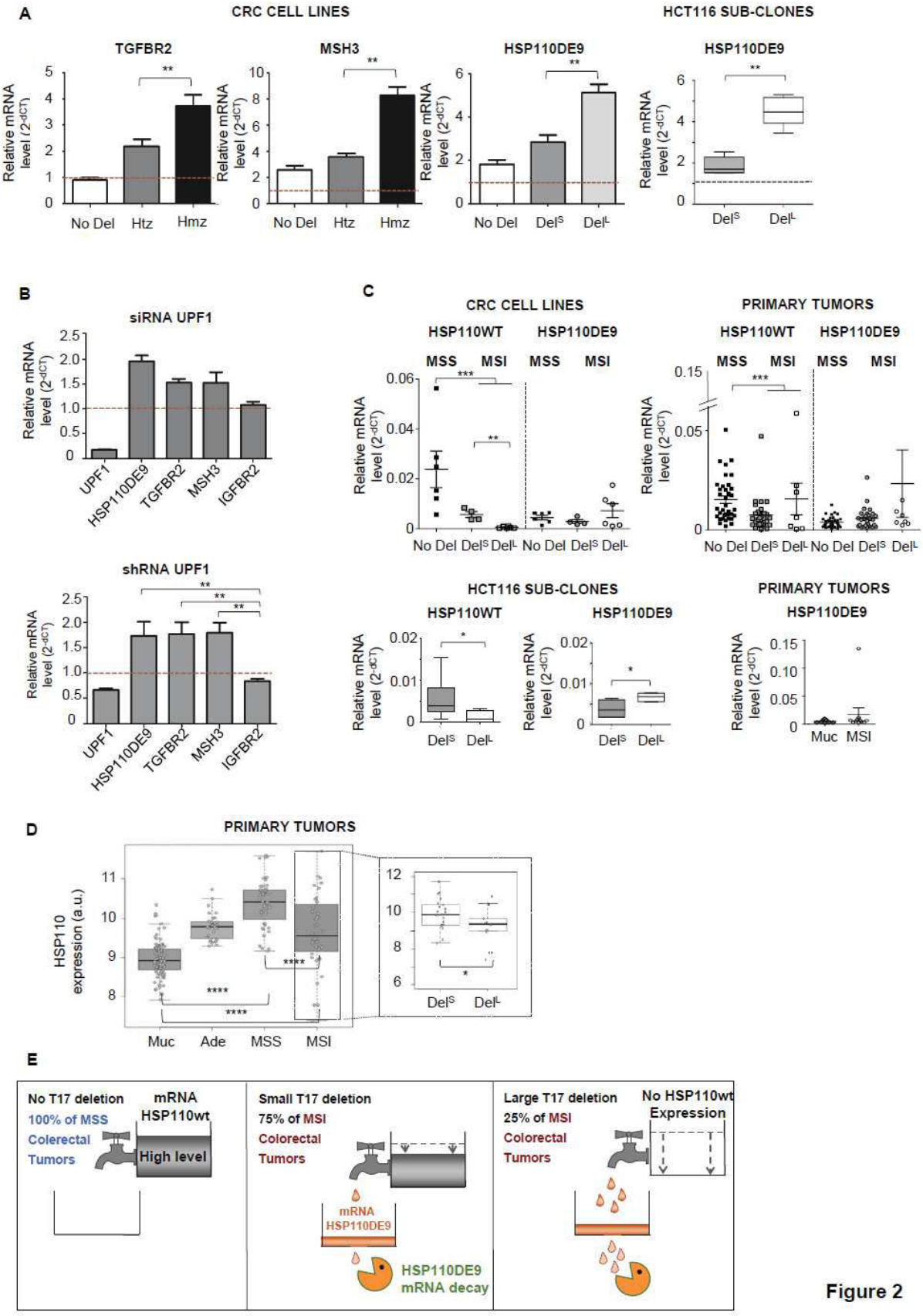

A
CRC CELL LINES
HCT116 SUB-CLONES
TGFBR2
MSH3
HSP110DE9
HSP110DE9
Relative mRNA level (2-dCT)
No Del
Htz
Hmz
DelS
DelL
B
siRNA UPF1
shRNA UPF1
UPF1
HSP110DE9
TGFBR2
MSH3
IGFBR2
C
CRC CELL LINES
PRIMARY TUMORS
HSP110WT
HSP110DE9
MSS
MSI
HCT116 SUB-CLONES
PRIMARY TUMORS
Muc
MSI
D
PRIMARY TUMORS
HSP110 expression (a.u.)
Muc
Ade
MSS
MSI
E
No T17 deletion
100% of MSS
Colerectal
Tumors
mRNA
HSP110wt
High level
Small T17 deletion
75% of MSI
Colorectal
Tumors
mRNA
HSP110DE9
HSP110DE9
mRNA decay
Large T17 deletion
25% of MSI
Colorectal
Tumors
No HSP110wt
Expression

Figure 2

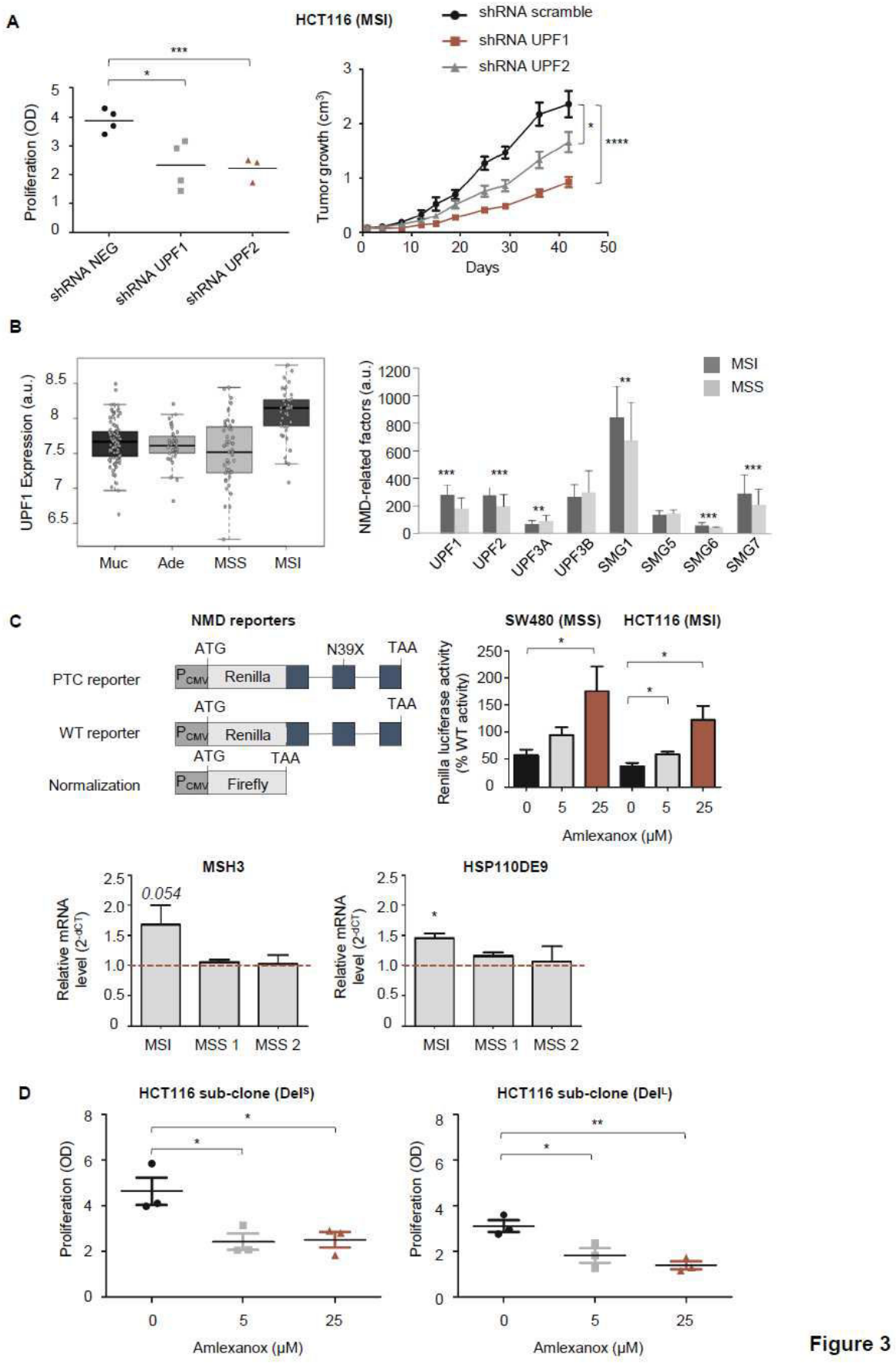

A
HCT116 (MSI)
shRNA scramble
shRNA UPF1
shRNA UPF2
Proliferation (OD)
shRNA NEG
shRNA UPF1
shRNA UPF2
Tumor growth (cm³)
Days
B
UPF1 Expression (a.u.)
Muc
Ade
MSS
MSI
NMD-related factors (a.u.)
MSI
MSS
UPF1
UPF2
UPF3A
UPF3B
SMG1
SMG5
SMG6
SMG7
C
NMD reporters
ATG
N39X
TAA
PTC reporter
WT reporter
Normalization
Renilla
Firefly
SW480 (MSS)
HCT116 (MSI)
Renilla luciferase activity (% WT activity)
Amlexanox (μM)
MSH3
HSP110DE9
Relative mRNA level (2^-dCT)
0.054
MSI
MSS 1
MSS 2
D
HCT116 sub-clone (Del^S)
HCT116 sub-clone (Del^L)
Proliferation (OD)
Amlexanox (μM)

Figure 3

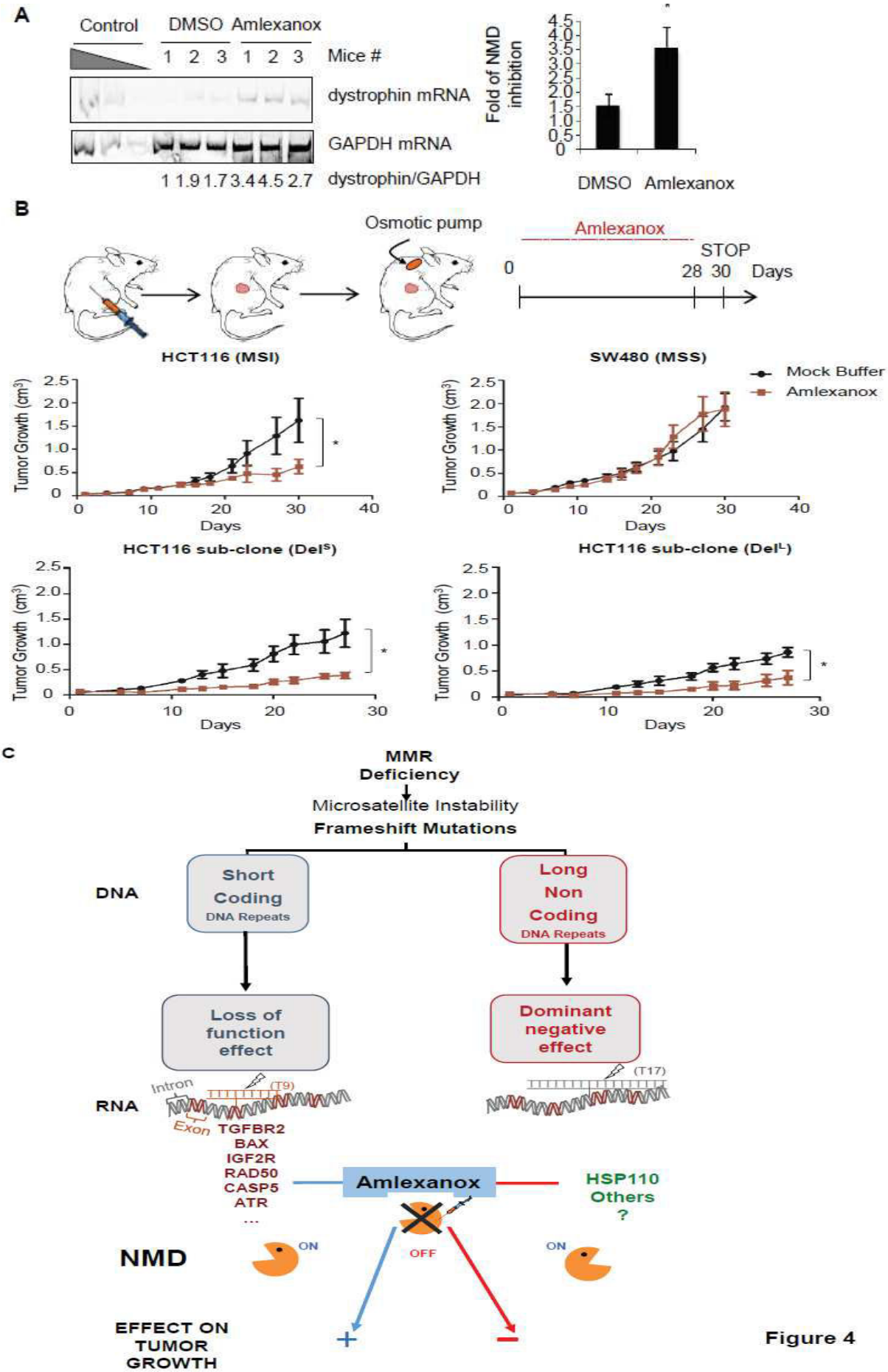

A
Control
DMSO Amlexanox
1 2 3 1 2 3
Mice #
dystrophin mRNA
GAPDH mRNA
1 1.9 1.7 3.4 4.5 2.7
dystrophin/GAPDH
Fold of NMD inhibition
DMSO Amlexanox
B
Osmotic pump
Amlexanox
STOP
0
28 30 Days
HCT116 (MSI)
SW480 (MSS)
Mock Buffer
Amlexanox
Tumor Growth (cm³)
Days
HCT116 sub-clone (Del^S)
HCT116 sub-clone (Del^L)
C
MMR Deficiency
Microsatellite Instability
Frameshift Mutations
DNA
Short Coding DNA Repeats
Long Non Coding DNA Repeats
Loss of function effect
Dominant negative effect
RNA
Intron
Exon
(T9)
(T17)
TGFBR2
BAX
IGF2R
RAD50
CASP5
ATR
...
Amlexanox
HSP110
Others
?
NMD
ON
OFF
ON
EFFECT ON TUMOR GROWTH
+
−

Figure 4

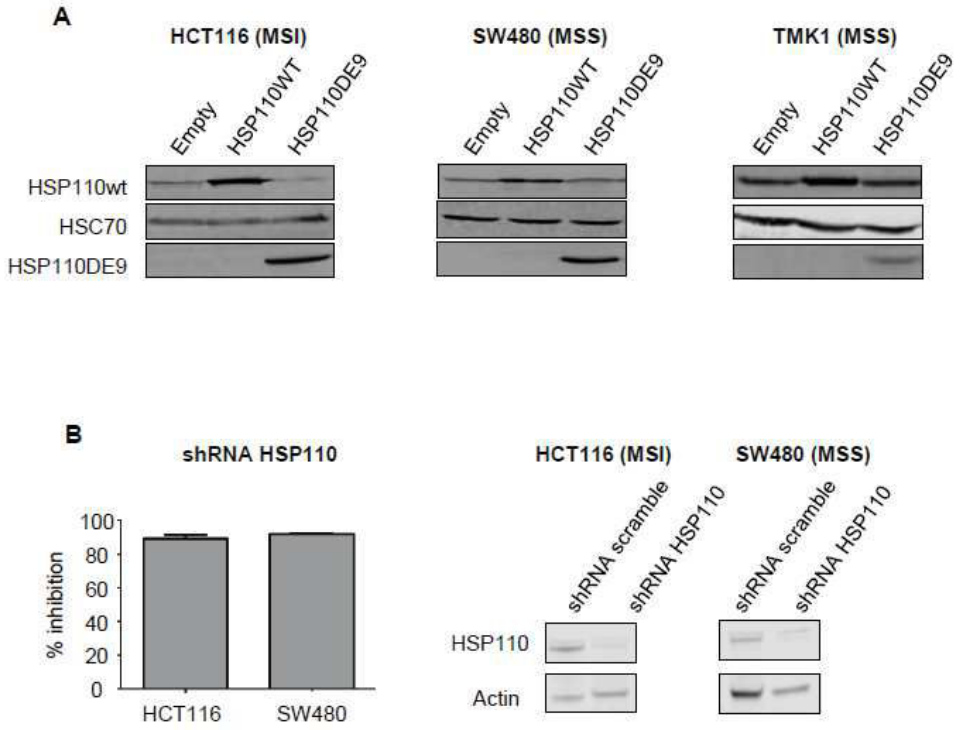
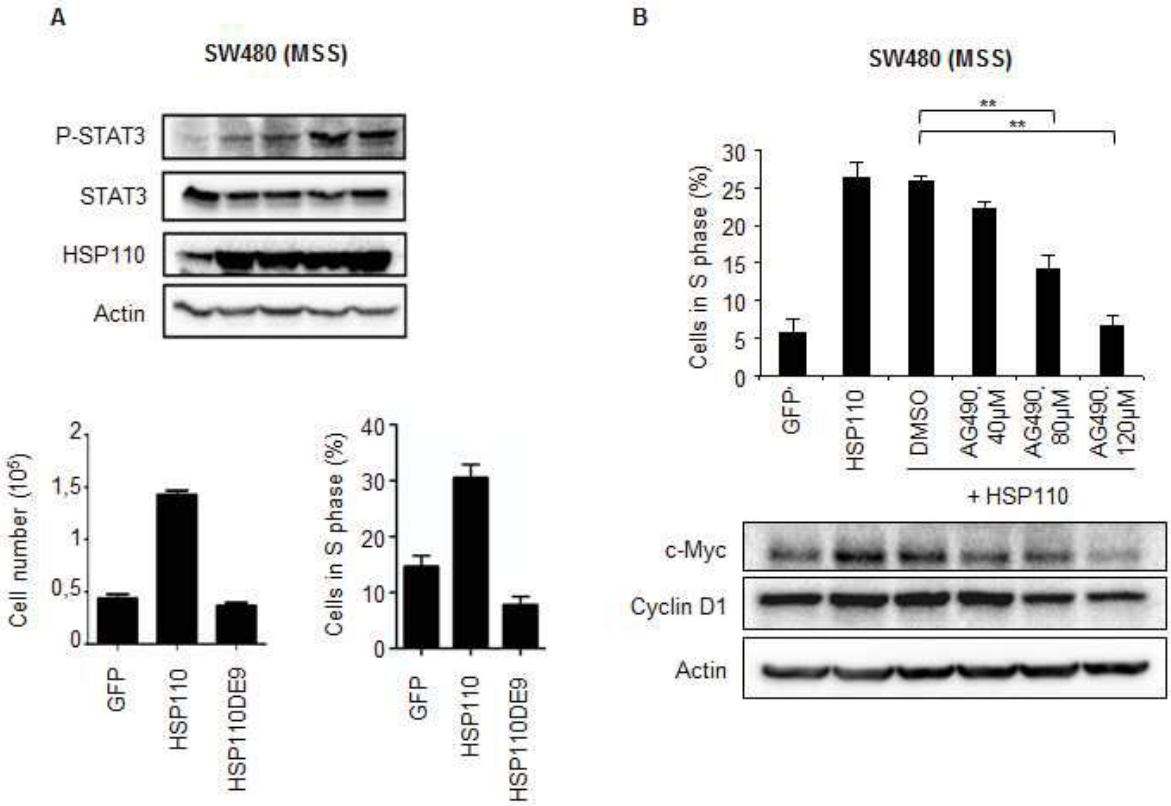

A
HCT116 (MSI)
SW480 (MSS)
TMK1 (MSS)
Empty
HSP110WT
HSP110DE9
HSP110wt
HSC70
HSP110DE9
B
shRNA HSP110
% inhibition
100
80
60
40
20
0
HCT116
SW480
HCT116 (MSI)
SW480 (MSS)
shRNA scramble
shRNA HSP110
HSP110
Actin

Figure S1


A
SW480 (MSS)
P-STAT3
STAT3
HSP110
Actin
Cell number (10⁶)
2
1,5
1
0,5
0
GFP
HSP110
HSP110DE9
Cells in S phase (%)
40
30
20
10
0
GFP
HSP110
HSP110DE9
B
SW480 (MSS)
**
**
Cells in S phase (%)
30
25
20
15
10
5
0
GFP
HSP110
DMSO
AG490, 40µM
AG490, 80µM
AG490, 120µM
+ HSP110
c-Myc
Cyclin D1
Actin

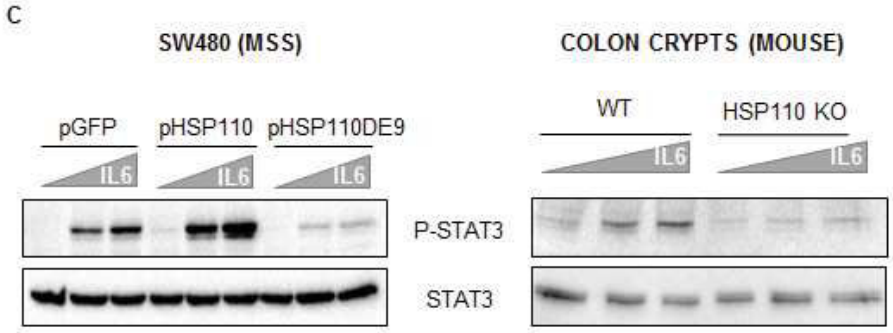
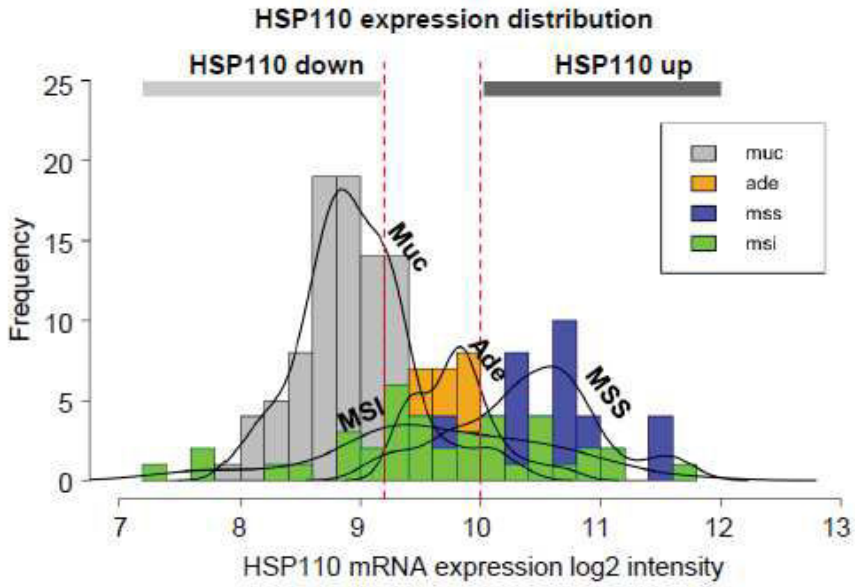

C
SW480 (MSS)
COLON CRYPTS (MOUSE)
pGFP
pHSP110
pHSP110DE9
IL6
IL6
IL6
WT
HSP110 KO
IL6
IL6
P-STAT3
STAT3

Figure S2

A


HSP110 expression distribution
HSP110 down
HSP110 up
muc
ade
mss
msi
Muc
Ade
MSS
MSI
Frequency
HSP110 mRNA expression log2 intensity

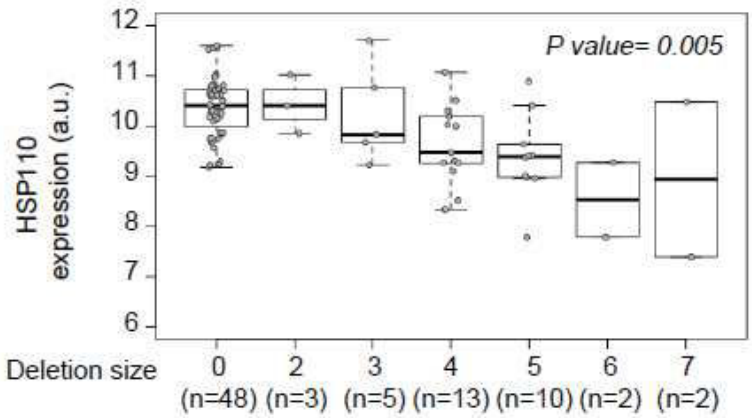

P value= 0.005
HSP110 expression (a.u.)
Deletion size
0 (n=48)
2 (n=3)
3 (n=5)
4 (n=13)
5 (n=10)
6 (n=2)
7 (n=2)

Figure S3

Figure Legend

Figure 1. Decrease in HSP110 activity is responsible for inhibition of MSI and MSS colon tumor growth in xenografts and modulates STAT3 activity. (A) Comparative analysis of tumor growth (mean tumor volumes) in xenografts derived from HSP110-mutated HCT116 sub-clones (see (Collura et al., 2014) for the procedure we used to isolate HCT116 sub-clones from the parental HCT116 cell line)**.** Ten mice per group. Mice were injected with HCT116 sub-clones displaying small ([Del^S]; n = 2 sub-clones, five mice engrafted with each clone) or large ([Del^L]; n = 2 sub-clones, five mice engrafted with each clone) T17 deletion. Data are means ± SEM. *** $p < 0.001$. **(B)** Comparative analysis of tumor growth (mean tumor volumes) in xenografts stably transfected with vectors expressing HSP110wt or HSP110DE9 protein. Experiments were performed with MSI (HCT116) and MSS (SW480) CRC cells and also with MSS gastric tumor cells (TMK1). Ten mice per group. Data are means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. **(C)** Comparative analysis of tumor growth (mean tumor volumes) in xenografts derived from HCT116 or SW480 CRC cells stably transfected with EBV-based empty vector or siHSP110 vector (shRNA) permitting long-term gene silencing. Ten mice per group. Data are means ± SEM. *** $p < 0.001$. **(D)** Left panel: Immunoblot analysis of P-STAT3, STAT3 and HSP110 in HCT116 sub-clones with either small (Del^S) or large (Del^L) deletions in the HSP110 T17 repeat. Actin, loading control. Middle panel: Immunoblot analysis of P-STAT3, HSP110 and HSP110DE9 in SW480 and HCT116 CRC cells, 48h after transfection with plasmids coding for HSP110-GFP and increasing concentrations of HSP110DE9-GFP. HSC70 serves as a loading control. Right panel: Immunoblot analysis of cyclin D1, c-Myc, Mcl1, Bcl-xL and P-STAT3 in SW480 CRC cells, 48h after transfection by plasmids coding for control-GFP, HSP110-GFP or HSP110DE9-GFP. HSC70 serves as a loading control. **(E)** Left panel: Representative images of HCT116 sub-clones (Del^S and Del^L) xenograft sections stained with P-STAT3 antibody by immunohistochemistry (experiments in 5x replicates). P-STAT3 expression is positive in Del^S cells whereas no expression of P-STAT3 was observed in Del^L cells. Magnification x100. Right Panel: Immunostaining in primary MSI colon tumors. Significant associations were observed for the expression of HSP110 and P-STAT3, and for the expression of HSP110 overall

and T17 deletion status in these tumors (upper panel). Representative images of primary colon tumors displaying double positive or double negative immunostaining are shown. Magnification x100. For left and right panel, the inserts correspond to a detail of the immunostaining. Magnification x200.

Figure 2. HSP110DE9 expression is censored due to Nonsense-mediated mRNA decay in MSI colon cancer cells and primary tumors. (A) Relative expression levels of *TGFBR2*, *MSH3* or *HSP110DE9* mRNAs determined by quantitative RT-PCR in CRC cell lines (left panel) or in HCT116 sub-clones (right panel: Del^S, n=2; Del^L, n=2) after cycloheximide [CHX] treatment (4h, 400µg/ml). CRC cell lines analyzed with *HSP110DE9* probes, [No Del] = SW480 and FET; [Del^S] = HCT116 and HCT8; [Del^L] = RKO and LS174T. CRC cell lines analyzed with *TGFBR2* probe, [No Del] = SW480 and FET; Heterozygote [Htz] = HCT8 and RKO; Homozygote [Hmz] = HCT116 and LS174T. CRC cell lines analyzed with *MSH3* probe, [No Del] = SW480 and HCT8; Heterozygote [Htz] = LS174T; Homozygote [Hmz] = HCT116. **(B)** Relative mRNA expression levels of candidate target genes for MSI (containing coding DNA repeats) determined by quantitative RT-PCR in HCT116 CRC cells transfected with siRNA-UPF1 (left panel) or shUPF1 (right panel). In HCT116, HSP110DE9 status is Del^S, *TGFBR2* and *MSH3* status are Hmz. *IGF2R* status is not mutated. **(C)** Upper left panel: Relative mRNA expression levels of HSP110wt and HSP110DE9 determined by quantitative RT-PCR in MSS and MSI CRC cell lines. No T17 deletion [No Del], n=6 CRC cell lines; small T17 Deletion [Del^S], n=4 CRC cell lines; large T17 Deletion [Del^L], n=6 CRC cell lines. Lower left panel: Relative mRNA expression levels of HSP110wt and HSP110DE9 determined by quantitative RT-PCR in HCT116 sub-clones. Del^S (n= 2 sub-clones); Del^L (n= 2 sub-clones). Upper right panel: Relative expression levels of HSP110wt and HSP110DE9 mRNAs by quantitative RT-PCR in MSS and MSI CRC primary tumors. No Del (MSS CRCs), n=36; Del^S (MSI CRCs), n=28; Del^L (MSI CRCs), n=7. Lower right panel: Relative expression levels of HSP110DE9 mRNA determined by quantitative RT-PCR in primary tumors and paired normal colonic mucosa. [Muc-MSS], n=6 samples; [Muc-MSI], n=11 samples. **(D)** Quantification of all HSP110 mRNAs regardless of the presence or absence of exon 9 using micro-arrays (normal colonic mucosa [Muc], n=95; adenoma [Ade], n=32; MSS colon carcinoma [MSS], n=48;

MSI colon carcinoma [MSI], n=40). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(E)** Proposed scheme for the molecular consequences of *HSP110* T17 deletions in colon tumors taking account of NMD activity. In contrast to MSS tumors, all MSI tumors have a deletion in the T17 *HSP110* sequence. In MSS CRCs, wild type HSP110 protein is highly expressed and the HSP110DE9 mutant is not produced. MSI colon tumors with small T17 deletions (≤ 4 pb, about 75% of all CRC MSI) still express wild type HSP110 protein at significant levels. In contrast, MSI colon tumors with large deletions (≥ 5 pb, about 25% of all CRC MSI) do not express wild type HSP110 anymore. In both these subclasses of MSI CRCs, endogenous expression of HSP110DE9 is maintained at low levels because this mutant mRNA is censored by NMD.

Figure 3. Inhibition of NMD using ShRNA targeted to UPF factors and Amlexanox in MSI and MSS colon cancer cells. (A) Left panel: Proliferation of HCT116 CRC cells stably transfected with shRNA-Control (scrambled), ShUPF1 or ShUPF2. Right panel: Comparative analysis of tumor growth (mean tumor volumes) in nude mice xenografted with HCT116 CRC cells transfected with the same shRNA-Control (scrambled), ShUPF1 or ShUPF2. Ten mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(B)** Microarray analysis of UPF1 expression (left panel: [normal Mucosa], n=95; [Adenoma], n=32; [MSS primary CRC], n=48; [MSI primary CRC], n=40) and other NMD-related factors (right panel). * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. **(C)** Schematic representation of the mammalian NMD reporter system used in this work (Boelz et *al*., BBRC, 2012). The NMD reporter gene consisted of an in-frame Renilla luciferase/β-globin fusion construct with (N39X, PTC) or without (WT) a nonsense mutation at codon 39 of the β-globin open reading frame (upper left panel). Co-expressed firefly luciferase activity was used to normalize the level of Renilla luciferase activity in MSI (HCT116) and MSS (SW480) CRC cells after 24H Amlexanox treatment at concentrations of 5 or 25 μM (upper right panel). Lower panel: Relative mRNA expression of *MSH3* (HMZ mutation) and *HSP110DE9* by quantitative RT-PCR in MSI (HCT116) and MSS (1: SW480 and 2: HT29) CRC cells after 24H Amlexanox treatment at concentration of 5 μM. * $p < 0.05$. Data are means ± SEM. **(D)** Proliferation assay for HCT116 (MSI) sub-clones ([Del^S], [Del^L]) treated once daily for a 4 days period with

Amlexanox at concentrations of 5 or 25 μM. All experiences were done in triplicates. Data are means ± SEM. * $p < 0.05$; ** $p < 0.01$.

Figure 4. Inhibition of NMD using Amlexanox in a dystrophin mouse model and in mice xenografted with HCT116 (MSI) and SW480 (MSS) CRC cells. (A) MDX mice harboring a nonsense mutation in exon 23 of the dystrophin gene were injected subcutaneously with 1.2mg of amlexanox/kg for 24 hours. The results for 3 mice injected with DMSO or 3 mice injected with amlexanox are shown. The 3 left lanes represent a serial dilution of RT from an untreated wild-type mouse. * $p < 0.1$. **(B)** Schematic representation of the protocol for treating mice with the NMD inhibitor amlexanox (upper panel). The osmotic pump contained either a mock buffer made with 50% DMSO and 50% PEG400, or amlexanox diluted in the mock buffer in order to deliver 0.15mg of amlexanox per day to each mouse during 28 days. Comparative analysis of tumor growth (mean tumor volumes) in mice treated with or without amlexanox. Eight mice per group. Experiments were performed with MSI (HCT116) and MSS (SW480 cell line) CRC cells (upper left and right panel) and with HCT116 sub-clones (Del^S and Del^L) (lower left and right panel). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. **(C)** Schematic representation of the role of NMD in MSI tumors. Numerous MSI-driven mutant PTC-mRNAs processed by NMD are generated due to frameshift truncating mutations in coding DNA microsatellites in MMR-deficient colon tumors. In contrast to that of HSP110, these mutations are supposed to be oncogenic by resulting in loss of function effects inactivating tumor suppressor genes (*e.g.* TGFBR2, BAX, IGF2R, RAD50, …). Although these mutant PTC-mRNAs are degraded by NMD, no resultant pathophysiological impact is expected since the corresponding mutant proteins have lost their function in the majority of cases and therefore do not have residual biological activity. In sharp contrast, HSP110DE9 mutant processing by the NMD is shown here to have biological negative consequences for the tumor.

Figure S1: (A) Immunoblot analysis of HSP110WT and HSP110DE9 overexpression in CRC (MSI: HCT116; MSS: SW480) and gastric (MSS: TMK1) cancer cell lines stably transfected with vectors expressing wild type HSP110 or HSP110DE9 mRNA. HSC70 serves as a loading control. **(B)** Left panel: Percentage of HSP110 inhibition in CRC cell

lines stably transfected with shRNA-control and shRNA-HSP110 (MSI: HCT116; MSS: SW480). Right panel: Immunoblot analysis of HSP110 in CRC cells stably transfected with shRNA-control and shRNA-HSP110. Actin serves as a loading control. Data are means ± SEM.

Figure S2: STAT3 pathway regulation by HSP110 (**A)** Upper panel: Immunoblot analysis of P-STAT3, STAT3 and HSP110 in SW480, 48H after transfection with plasmids coding for HSP110 (HA- and GFP-tagged, wells 2, 3 and 4, 5 respectively). Actin serves as loading control. Lower panel: The number of SW480 cells and percentage of cells in the S phase after BrdU incorporation and 7-AAD staining were determined 48H after transfection with plasmids coding for control-GFP, HSP110-GFP or HSP110DE9-GFP. n=5. * $p<0.05$; **** $p<0.0001$. Data are means ± SEM. **(B)** Upper panel: Percentage of GFP positive SW480 cells in the phase S assessed by BrdU incorporation and 7-AAD staining, 48H after transfection with plasmids coding for control-GFP, HSP110-GFP and treated during the last 24 hour with or without increasing doses of AG490 (40, 80, 120 µM). n=4. Lower panel: Immunoblot analysis of c-myc and cyclin D1. Actin serves as loading control. ** $p < 0.01$. Data are means ± SEM. **(C)** Left panel: Immunoblot analysis of P-STAT3, STAT3 in SW480, 48H after transfection with plasmids coding for control-GFP, HSP110-GFP or HSP110DE9-GFP and treated during 30 minutes with or without IL-6 (10 or 100 ng/mL). Right panel: Immunoblot analysis of P-STAT3 in mouse colon crypt biopsies isolated from wild type or HSP110 KO mice and treated ex vivo during 30 minutes with or without IL-6 (10 or 100 ng/mL). STAT3 serves here as a loading control. n=4. One representative experiment is shown here.

Figure S3: Quantification study of all HSP110 mRNAs. (A) Left panel: Densities and bar plots of HSP110 log2 intensities in normal colonic mucosa (Muc), adenomas (Ade), MSS tumors (MSS) and MSI tumors (MSI). Right panel: Boxplot of HSP110 log2 intensity levels according to HSP110 T17 deletion size.

EXPERIMENTAL PROCEDURES

Primary colon tumor samples and CRC cell lines. CRC cell lines were purchased from the American Type Culture Collection. HCT116 sub-clones were obtained using MoFlo Astrios (Beckman Coulter, Paris, France), spotting 1 cell/well in 96-well plates containing 200 µL of Dulbecco's modified Eagle media. All cells were cultured in Dulbecco's modified Eagle media as described. Primary tumors and normal colonic tissues were obtained from patients undergoing surgery in our hospital (Hôpital Saint-Antoine, Paris, France). MSI status was determined as described previously (Buhard et al., 2006).

HSP110 T17 deletion analysis and Real-Time quantitative RT-PCR analysis. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen). T17 deletion status was determined as previously described (Dorard et al., 2011). Total RNA was extracted with an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA integrity was evaluated on a 2100 Bioanalyzer using the RNA 6000 Nano LabChip kit (Agilent) for all primary tumor samples. Only samples with RIN > 5 were used. Complementary DNAs were synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems). For quantitative RT-PCR, we used the Applied SDS Biosystems analysis software. Expression values of *HSP110wt* and *HSP110DE9* transcripts were calculated relative to RPLP0 ubiquitous RNA, and expression values for *TGFBR2, MSH3, BAX, IGF2R* and *GAPDH* were calculated relatively to 18S ubiquitous RNA. Primers and internal probes for *HSP110wt* and *HSP110DE9* were as described earlier (Dorard et al., 2011). Primers and internal probes for *TGFBR2, MSH3, BAX, IGF2R, GAPDH, 18S* and *RPLP0* were those proposed by Applied Biosystems (TaqMan gene expression assays). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min.

Cycloheximide and Amlexanox treatment *in vitro*. Cells seeded into 6-well culture plates ($2x10^5$ cells per well) in DMEM media supplemented with 10% FCS containing 10 $U.ml^{-1}$ penicillin G and 100 $\mu g.ml^{-1}$ streptomycin were treated with Cycloheximide (400 $\mu g.ml^{-1}$, Sigma-Aldrich) for 4h or with 5 micromolar of amlexanox (5, 25 micromolar) for 24 hours prior to cell harvest and RNA extraction using RNeasy Mini Kit (Qiagen®).

Transfection with siRNA. The HCT116 CRC cell line was seeded into 6-well culture plates ($1x10^5$ cells per well) and transiently transfected with 50 nM of siRNA directed against UPF1 or with non-specific siRNA (Thermo Fisher) using the Dharmafect reagent (Thermo Fisher) according to manufacturer's instructions. A siRNA directed against GAPDH (Thermo Fisher) was used as a transfection control. Cells were collected for total RNA extraction 48h post-transfection. Each transfection experiment was performed in triplicate.

Transfection with shRNA and xenograft. Cloning of pEBVsiRNA vectors and establishment of silenced cells were performed as described previously (Biard, 2007). We used the DSIR program for designing shRNA sequences targeting the *HSP110* gene (Vert et al., 2006). RNAi sequences targeting the HSP110 (NM_006644) mRNA stretched over nucleotides 179-197 (pBD3226), 292-310 (pBD3227) or 406-424 (pBD3228). As control we used cells carrying the pBD650 plasmid that expressed an inefficient shRNA sequence.

The HCT116 CRC cell line was seeded into a 10 cm petri dish ($6x10^5$ cells per well) in the presence of 10µg of shRNA directed against UPF1, UPF2 or non-specific shRNA (Thermo Fisher) using the Dharmafect reagent (Thermo Fisher) according to manufacturer's instructions. An shRNA directed against GAPDH (Thermo Fisher) was used as a transfection control. After 72 hours, the cell medium was supplemented with puromycin (3µg/mL). After several days, sub-clones were selected for each shRNA. *In vivo*, $10x10^6$ cells of each HCT116 sub-clone transfected with shRNA were injected subcutaneously into the flank of Nude mice (Charles River Laboratories, Wilmington, USA). The tumor size was measured 3 times per week during 42 days.

EBV-based vectors construction, stable transfection and xenograft. We introduced specific siHSP110 sequence into hygromicin-resistant pEBV plasmids (REF). Cells were plated 24h before transfection with JetPrime (Ozyme) according to the manufacturer's recommendations. 24h later, cells were trypsinized and seeded in culture medium supplemented with hygromicin (125 µg/ml for HCT116 cell line or 250 µg/ml for SW480). After several days, $10x10^6$ cells of HCT116 and SW480 cell lines transfected with shRNA were injected subcutaneously into the flank of Nude mice (Charles River Laboratories, Wilmington, USA). The tumor size was measured 3 times per week during 32 and 25 days respectively.

Cell Proliferation. Proliferation rates were assessed in culture using WST-1 (Roche, Mannheim, Germany). $2x10^4$ cells were plated per well in 24-well plates in 2mL of media. WST-1 reagent was added and incubated for 4 hours at 37°C at the end of the proliferation test. The absorbance was measured at 450nm and the reference wavelength was 750nm.

Transient cell transfection and treatments. $1.2x10^5$ SW480 cells or $2.5x10^5$ HCT116 cells were cultured in a 12-well plate for 24h. Cells were then transfected with 1µg of plasmid coding for either GFP, GFP-HSP110 or GFP-HSP110DE9 using HP Xtreme gene DNA transfection reagent (Roche, Boulogne-Billancourt, France) according to the manufacturer's instructions. In some experiments**,** cells were treated by the Janus kinase 2 protein inhibitor AG490 (Millipore, Molsheim, France) 24h after transfection for 24h. To induce STAT3 activation, human cell lines or mouse colon crypts were treated respectively with human (Life technologies, Saint-Aubin, France) or mouse (Miltenyi, Paris, France) IL-6 (10 or 100 $ng.mL^{-1}$).

Cell cycle analysis. Cell cycle was analyzed using the APC-BrdU Flow kit from BD Pharmingen (Franklin Lakes, USA). Briefly, cells were incubated with BrdU (10µM, 60 min), then washed in PBS, fixed with BD cytofix/cytoperm solution (15 min, RT) and permeabilized with Cytoperm/Permeabilisation (10 min). After wash and 5 min incubation with BD Cytofix/Cytoperm, cells were incubated for 1 hour at 37°C with DNase ($300µg.ml^{-1}$). Cells were then washed and incubated with APC-labeled anti-BrdU antibody (20 min. RT). 7-AAD was used to stain total DNA and cell cycle analysis was performed using a LSRII flow cytometer Becton Dickinson, Franklin lakes, USA).

Stable cell transfection and xenografts. HCT116, SW480, and TMK1 cell lines were transfected with an Epstein-Barr virus-based vector construction coding either for HSP110wt, HSP110DE9 or an empty vector, as previously described (Collura et al., 2014). $10x10^6$ cells of each cell line transfected with plasmid were then injected subcutaneously into the flank of

Nude mice (Charles River Laboratories, Wilmington, USA). The tumor size was measured 3 times per week during 29 days (SW480 cell line) or 38 days (HCT116 and TMK1 cell lines).

Immunoblot analysis. Cells were harvested, washed in PBS and then lysed on ice in lysis buffer (150mM NaCl, 50 mM Tris pH 6,8, 10 mM NaF, 1mM DTT, 1% Triton X-100) in the presence of protease (Roche, Boulogne-Billancourt, France) and phosphatase (Sigma-Aldrich, Lyon, France) inhibitors. Proteins were separated and transferred following standard protocols before analysis with a chemiluminescence detection kit (Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies used for immunobloting were from Cell signalling (Danvers, USA) directed against Bcl-xL (2764S), c-Myc (9605S), Cyclin D1 (2926S), P-STAT3 (9145S) and STAT3 (9139S); from Santa Cruz biotechnologies directed against Mcl-1 (sc-819), HSC70 (sc-7298), HSP110 (sc-6241), GFP (sc-8334); and from Sigma directed against anti-actin (A1978-200UL).

Cytoplasmic and nuclear extracts were obtained using the « NE-PER Nuclear and Cytoplasmic Extraction reagents » kit from Thermo Scientific (Waltham, USA).

Immunohistochemistry of STAT3 in HCT116 sub-clones xenograft. $10x10^6$ HCT116 sub-clone cells were injected subcutaneously into the right flank of NOD/SCID mice (Charles River Laboratories, Wilmington, USA). Tumor growth was followed every second day for 3 weeks. Mice were sacrificed when tumors reached $800mm^3$. The mice were treated according to the guidelines of the Ministère de la Recherche et de la Technologie, France. Tumor sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Antigens were then unmasked and the slides incubated in pH 8,0 EDTA buffer (30 min, 95°C), cooled for 30 min, washed twice in PBS for 3 min and treated with 3% H_20_2-PBS for 15 minutes in order to inhibit endogenous peroxidases. After washes in PBS, the slides were saturated for 25 min in 3% BSA PBS. 150µL of primary P-STAT3 antibody (1:50) was deposited onto slides and left in a humidified chamber overnight at 4°C. After washing in PBS, secondary antibody (8114P, Cell signalling) was added for 30 minutes at room temperature. Slides were washed twice for 5 minutes in PBS and revealed using Novared kit (Vector, Burlingame, USA). Slides were washed twice in water for 5 minutes and counterstained with 10% Meyer's hematoxylin. After one wash in water, slides were dehydrated in 100% ethanol and in xylene for 30 seconds each. The slides were then observed using the Cell Observer station (Zeiss, Germany).

Immunohistochemistry of STAT3 and HSP110 in primary colon tumors. Briefly, 4 µm sections of paraffin-embedded tissue samples were cut onto silane-treated Super Frost slides (CML, Nemours, France) and left to dry at 37°C overnight. Tumor sections were deparaffinized in xylene and rehydrated in pure ethanol. Before immunostaining, antigen retrieval was performed by immersing sections in citrate buffer (pH 6.0) for HSP110 or in pH 8.0 EDTA buffer for P-STAT3 (15 min at 95°C), washed twice in PBS for 3 min and treated with 3% H_20_2-PBS for 15 minutes in order to inhibit endogenous peroxidases. After washing in PBS, slides were saturated for 25 min in 3% BSA PBS. Sections were then incubated for 1 hour at room temperature with antibody to HSP110 (dilution 1/1200; clone 5812, Leica Biosystems) and overnight at 4°C in a humidified chamber with antibody to P-STAT3 (dilution 1/70, clone D3A7, Ozyme). After washing in PBS, secondary antibody (8114P, Cell signaling) was added for 30 minutes at room temperature. Slides were washed twice for 5 minutes in PBS and

revealed using Novared kit (Vector, Burlingame, USA). Slides were washed twice in water for 5 minutes and counterstained with 10% Meyer's hematoxylin. After one wash in water, slides were dehydrated in 100% ethanol and then in xylene for 30 seconds each.

Measurement of *Dystrophin* mRNA levels by RT-PCR. Oligonucleotide sequences used for GAPDH: 5'-CATTGACCTCACTACATGG-3' and 5'-GCCATGCCAGTGAGCTTCC-3', ; for dystrophin 5'-TGGTGGGAAGAAGTAGAGGACTG-3' and 5'-GCAGTGCCTTGTTGACATTGTTCAG-3'.

Measurement of HSP110 and NMD factor mRNA expression by transcriptome microarray. A large series of 40 MSI tumors, 48 MSS tumors and 42 normal colonic mucosa were screened for mRNA expression using Affymetrix U133Plus chips as previously described (Marisa et al., 2009) (data partly in GSE33582 data set). Additional adenoma and mucosa samples were added from GSE8671 and GSE4183 data sets. Data were normalized together by Robust Multi-array Average normalization (R package affy). Associations with annotations were assessed by ANOVA or t-test (R package stats). Differential expression of NMD factors between tumor types were assessed by moderated t-test and FDR multiple testing correction (R package limma).

***In vivo* effect of amlexanox.** 5 week old nude mice were injected with 10^7 cells (SW480 or HCT116) subcutaneously in the right back side. Around 5 days later when the tumor reached 4mm, an osmotic pump was introduced under the skin of each animal at the left back side. Osmotic pumps contained either a mock buffer made of 50% DMSO and 50% PEG400, or mock buffer with amlexanox. Tumor size was measured 3 times per week during 30 days. MDX mice harbouring a nonsense mutation in exon 23 of the dystrophin gene were injected subcutaneously with 1.2mg of amlexanox/kg for 24 hours prior to collection of back leg muscles, extraction of RNA and performing quantitative RT-PCR.

Luciferase Reporter Assays. The Renilla luciferase-based NMD reporters were a gift from Dr. Andreas Kulozik (University of Heidelberg, Germany). HCT116 and SW480 cells were transfected with NMD reporters using lipofectamine (Invitrogen) as described (REF: Boelz S. et al 2006 BBRS). 24 hours after transfection, cells were incubated for 24 hours with 5 or 25 micromolar of amlexanox. Luciferase assays were performed with the Dual Assay System (Promega) using a Tecan Luminometer (Infinite F200 Pro).

Article 2: Extracellular HSP110 from colorectal cancer cells skew macrophages polarization

Les cellules immunitaires jouent un rôle important dans le contrôle du développement des cancers et notamment dans le cancer colorectal. Les macrophages selon leurs phénotypes peuvent inhiber ou promouvoir leur développement. Les cellules cancéreuses colorectales sécrètent différents facteurs capables d'influencer cette polarisation mais ceux-ci ne sont pas clairement identifiés. Les HSP peuvent être sécrétées et ont alors des fonctions immunologiques. HSP27 est ainsi capable de polariser les macrophages vers un phénotype M2 dans le cancer du sein. Au cours de ce second projet, nous nous sommes intéressés au rôle potentiel que pourrait jouer la sécrétion d'HSP110 dans le cancer colorectal sur la polarisation des macrophages.

Extracellular HSP110 skews macrophages polarization in colorectal cancer

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Running title: Extracellular HSP110 effect on macrophages.

Keywords: heat-shock protein, cancer, macrophage, polarization, toll-like receptor.

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Abstract

HSP110 is induced by different stresses and, through its anti-apoptotic and chaperoning properties, helps the cells to survive these adverse situations. In colon cancers, HSP110 is abnormally abundant. We have recently showed that colorectal cancer patients with microsatellite instability (MSI) had an improved response to chemotherapy because they harbor an HSP110 inactivating mutation (HSP110DE9). In this work, we have used patients' biopsies and human colorectal cancer cells grown *in vitro* and *in vivo* (xenografts) to demonstrate that 1) HSP110 is secreted by colorectal cancer cells and that the amount of this extracellular HSP110 is strongly decreased by the expression of the mutant HSP110DE9. 2) Supernatants from colorectal cancer cells overexpressing HSP110 or purified recombinant human HSP110 (LPS-free) affect macrophages differentiation/polarization by favoring a pro-tumor, anti-inflammatory, profile. 3) Conversely, inhibition of HSP110 (expression of siRNA, HSP110DE9 or immunodepletion) induced the formation of macrophages with a cytotoxic, pro-inflammatory, profile. 4) Finally, this extracellular HSP110 effect on macrophages seems to implicate the TLR4. These results, together with the fact that colorectal tumor biopsies with HSP110 high were infiltrated with macrophages with a pro-tumoral profile while those HSP110 low were infiltrated with cytotoxic profile macrophages, suggest that extracellular HSP110 function on macrophages may also contribute to the bad patient's outcome associated to HSP110 expression.

Introduction

Colorectal cancer (CRC) is a molecularly heterogeneous disease that can be subdivided into several molecular subtypes (Aaltonen et al., 1993). Approximately 15 to 20% of CRC harbor widespread microsatellite instability at DNA repeats (MSI) due to mismatch repair deficiency, in contrast to the majority of these tumors showing microsatellite stability (MSS)(Ionov et al., 1993; Thibodeau et al., 1993). MSI CRCs displayed particular morphologic features, including greater predilection for the right colon, mucinous histology, low metastatic power and poorer differentiation. They have been consistently reported to show an improved prognosis and a different response to chemotherapeutic agents. The molecular mechanisms of the peculiar MSI CRC pathophysiology have started to be uncovered recently. Notably, we have recently highlighted the important role of heat shock protein-110 (HSP110) (Collura et al., 2014; Dorard et al., 2011; Duval et al., 2011). HSPs are a set of highly conserved proteins whose expression is induced in response to a wide variety of physiological and environmental stress (Doyle et al., 2013; Saibil, 2013). They are often overexpressed in cancer cells and contribute to cancer resistance and apoptosis (Ciocca et al., 2013; Jego et al., 2013).

HSP110 is a high molecular weight chaperon that accumulates abnormally in CRC cells and whose expression correlates with metastasis and poor prognosis (Slaby et al., 2009). We have previously shown that a T_{17} mononucleotide repeat located in intron 8 of *HSP110* was systematically mutated in MSI CRC cell lines and primary tumors (Dorard et al., 2011). The shortening of this repeat in tumor DNA correlated with increased synthesis of an aberrant *HSP110* transcript due to exon 9 skipping (HSP110DE9) to the detriment of wild-type *HSP110* mRNA. MSI patients with large T17 deletions (low HSP110/high HSP110DE9) have significantly longer relapse-free survival (RFS) compared to those with small T17 deletions (high HSP110/low HSP110DE9) (Collura et al., 2014). Furthermore, MSI CRC patients with small T17 deletion (high HSP110 expression/low HSP110DE9) seemed to do not significantly differ in their RFS compared to MSS CRC patients, suggesting a strong dependency of CRC cells towards HSP110. Accordingly to this clinical data, our studies in vitro have demonstrated that

HSP110DE9 acts as a dominant negative mutant that binds to HSP110 wild type impairing its cellular localization and its ability to interact with other chaperones (Collura et al., 2014; Dorard et al., 2011). HSP110DE9 completely abrogates HSP110 chaperone activity and cytoprotective function. *In vitro*, HSP110DE9 expression sensitized colon cancer cells, in a dose dependent manner, to anticancer agents such as oxaliplatin and 5-fluorouracil.

Immune control of tumors is a well-established mechanism involved in various cancer progression, including CRC where tumor-infiltrating lymphocytes correlates inversely with tumor stage (Galon et al., 2006; Tosolini et al., 2011). Infiltration of activated CD8+ lymphocytes within and around the tumor stroma contributes to a better prognosis. In particular, MSI CRC patients have a higher number of tumor-infiltrating lymphocytes compared to MSS CRC and, besides HSP110, this may also explain the improved prognosis of MSI CRC patients when compared to MSS patients (Boissiere-Michot et al., 2014). Tumor-associated macrophages are also abundant tumor-infiltrating cells. In general, tumor-associated macrophages can be found within or surrounding various tumors where they either promote tumor progression, angiogenesis, migration of tumor cells and T helper 2 responses (so-called M2 or alternative macrophages), or, conversely, they promote resistance to tumors, inflammatory responses and T helper 1 responses (so-called M1 or classical macrophages) (Murray et al., 2014; Noy and Pollard, 2014). Although macrophages have been found in colorectal tumor sections (Edin et al., 2012; Forssell et al., 2007), the correlation of their abundance with the prognosis of MSI neoplasms is not clear yet.

Extracellular HSPs are described as damage associated molecular pattern proteins (DAMPs) with immunogenic properties. Extracellular HSPs such as HSP27 or HSP70 have been described to influence immune control of tumors, sometimes through immunosuppressive cells (Banerjee et al., 2011; Chalmin et al., 2010; Laudanski et al., 2007). HSP110 has been described to inhibit immune activation of dendritic cells through scavenger receptor binding (Qian et al., 2011). However, hardly anything is

known about the immune function of HSP110 in cancer. Here we have addressed the impact of extracellular HSP110 on macrophages profile in colorectal cancer.

Materials and methods

Cell culture and macrophages differentiation

CRC cell lines (HCT116 and SW480) were purchased from ATCC (Molsheim, France). C22 subclone derived from HCT116 cell line was previously described (reference 4). All cell lines were cultivated in DMEM supplemented with 10% FBS (Lonza, Amboise, France). Monocytes from human peripheral blood were obtained from healthy donors with informed consent and purified using CD14 microbeads labeling and magnetic cell sorting (Miltenyi Biotec, Paris, France) following manufacturer's instructions. 0.5 10^6 monocytes were incubated with 500 µL of CRC cell lines supernatant for 72h in a 24-wells plate in the presence of macrophage colony-stimulating factor (M-CSF, 100 ng/mL, Miltenyi Biotec) and then characterized. To analyze secreted cytokines, macrophages were incubated for an additional 24h in the presence of LPS (10ng/mL). In some experiments, recombinant HSP110 produced in HEK293 cells (OriGene Technologies, Rockville, MD) was added to the culture at 600 ng/mL. For receptor blocking experiment, isolated CD14+ monocytes (5.10^5) were preincubated for 30 min. with antibodies directed against TLR2 (5µg; MAB2616, R&D systems, Minneapolis, USA), TLR4 (10µg; AF1478, R&D systems) or SR-A (10µg; AF2708, R&D systems).

CRC Supernatant preparation

2.5 10^5 HCT116 or HCT116-C22 were cultured in a 12-wells plate for 72h in DMEM supplemented with 10% FBS. The supernatant was then harvested and centrifuged at 450 g for 5 minutes. For immunoblot experiments, culture medium was replaced after 72 h of culture by DMEM w/o serum for additional 8h and then concentrated using Amicon Ultra centrifugal filters (UFC501096, Merck Millipore, Molsheim, France) according to the manufacturer's instructions.

siRNA and Cell transfection

For plasmid transfection, 1.2 10^5 SW480 or 2.5 10^5 HCT116 were implanted and cultured in a 12-wells plate for 24h. Cells were then transfected with 1µg of plasmid encoding GFP, GFP-HSP110 or GFP-

HSP110DE9 using HP Xtreme gene DNA transfection reagent (Roche, Boulogne-Billancourt, France) according to the manufacturer's instruction. 48h hours later, the supernatant was collected and centrifuged at 450g for 5 minutes. For immunoblotting, culture media were replaced after 48h of transfection with DMEM w/o serum for additional 8h and then concentrated using Amicon Ultra centrifugal filters (UFC501096, Merck Millipore) according to the manufacturer's instructions.

For siRNA transfection, 2.5 10^5 SW480 or 0.5 10^6 HCT116 cells were seeded into a 6-wells plate and cultivated for 24h in DMEM 10% SVF. Cells were then transfected with 50 pmol of control siRNA (ON-TARGETplus Non-targeting Pool, D-001810-10-05) or HSP110 siRNA (ON-TARGETplus HSPH1 siRNA, L-004972-00-0005) from Dharmacon (GE Heathcare, Velizy, France), with Lipofectamine RNAiMAX Reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Media were replaced after 6h of incubation with transfection reagent and 24h after transfection. Media were then collected 72h after transfection and centrifuged at 450g for 5 minutes.

Xenograft model

Nude mice were purchased from Charles River Laboratories (Wilmington, USA) and housed in specific pathogen-free conditions. 10 10^6 HCT116 or HCT116-C22 were injected s.c. into the right flank of each mice. Tumor growth was then followed every other day for 3 weeks. Mice were sacrified when tumors reached 800mm^3. Tumors were collected and included in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Torrance, CA). The mice were treated according to the guidelines of the Ministère de la Recherche et de la Technologie, France.

Flow cytometry analysis

Macrophages were harvested, washed once in PBS and incubated 20 min with antibody directed against HLA-DR (561224, BD Horizon), CD163 (556018, BD Bioscience) and CD206 (550889, BD bioscicences) at 4°C. Cells were then washed twice in PBS and analyzed using a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, USA). For apoptosis determination, adherent and non-adherent cells

were harvested and stained with Annexin V-FITC and 7-AAD (BD Pharmingen, Franklin Lakes, USA) according to manufacturer's recommendation.

Immunodepletion

1.2 10^5 SW480 or 2.5 10^5 HCT116 were seeded and cultivated for 24h in a 12-wells plate. Cells were washed and 400µL of media without serum was then added for 8 hours. Supernatants were then harvested and centrifuged at 450g for 5 minutes. One ml of supernatant was then incubated overnight at 4°C under rotation with 2µg of control antibody (sc-2027, Santa Cruz Biotechnology, Dallas, TX, USA) or directed against HSP110 (sc-6241, Santa Cruz Biotechnology). 25µL of Protein A-agarose beads (Millipore, Billerica, MA) were added for 1h30 under rotation at 4°C. Supernatants were collected and stored at -20°C. Prior to incubation with monocytes, the immunodepleted supernatant was supplemented with FBS (to a final concentration of 10%). For immunoblot experiments, beads bound to HSP110 were washed 3 times with PBS and heated for 5 minutes at 95°C in laemmli buffer.

T lymphocytes proliferation assay

T Lymphocytes from peripheral blood of healthy donors were purified using pan T-Cells isolation kit (Miltenyi Biotec). T lymphocytes were then stained by using Cell Trace Violet (Invitrogen), according to the manufacturer's procedure. 10^5 T cells were then incubated for 3 days with 10.10^3 or 20.10^3 macrophages. T-cell division was detected by flow cytometry with an LSRII cytometer (BD Biosciences) and analyzed using ModFit software.

Cytokine and NO quantification

Cytokines secretion by macrophages upon LPS stimulation was determined using the MILLIPLEX MAP Kit "Human high sensitivity T Cell Magnetic Bead Panel (HSTCMAG-28SK, Millipore, Billerica, MA) according to the manufacturer's instructions. Cytokines secreted by CRC cell lines were analyzed using the Human Cytokine Array Panel A according to the manufacturer's instructions (ARY005, R&D

Systems). NO production was evaluated through nitrite measurement using The Griess Reagent System (Promega, Madison, WI).

Cell lysis and Immunoblotting

Cells were harvested, washed in PBS and then lysed on ice in lysis buffer (150mM NaCl, 50 mM Tris pH 6,8, 10 mM NaF, 1mM DTT, 1% Triton X-100) in the presence of protease inhibitors (Roche, Boulogne-Billancourt, France). Cell lysates or concentrated culture media were mixed to laemmli buffer. Proteins were separated and transferred following standard protocols before analysis with a chemiluminescence detection kit (Santa Cruz Biotechnology). Primary antibodies used for immunoblotting were from Santa Cruz biotechnologies directed against HSP110 (sc-6241), HSC70 (sc-7298), from Sigma (Lyon, France) for anti-actin (A1978-200UL), from abcam (Paris, France) for anti-HSP90α (ab59459) and from Enzo Life Sciences (Lyon, France) for anti-HSP70 (ADI-SPA-810) and anti-HSP27 (ADI-SPA-803).

Immunofluorescence

Tumor sections from xenograft were fixed 10 min. in cold acetone. Slides were dried and rehydrated in PBS for 5 min. twice. Slides were then saturated 20 min. in 3% BSA and 2% goat serum in PBS. Slides were incubated overnight at 4°C with (1:100) primary antibody directed against Arginase-1 (sc-20150, Santa Cruz Biotechnology) or NOS2 (sc-651, Santa Cruz Biotechnology), and F4/80 (MCA4971, AbD Serotec, Colmar, France). Slides were washed three times for 5 min. in PBS and incubated for 10 min. with a blocking reagent (R37107, molecular probes, Saint Aubin, France). (1:1000) Secondaries antibodies (Invitrogen) were added for 45 min. at room temperature. Slides were washed 3 times in PBS for 5 min. and mounted with ProLong (Thermo Fisher Scientific, Waltham, MA, USA)

Isolated CD14+ monocytes were incubated 30 min. in 10% RPMI with HSP110-Flag recombinant protein (500 ng / 5.10^5 monocytes). Cells were washed once and incubated for 30 min. at room temperature with (1:100) primary antibodies directed against TLR4 (sc-30002, Santa Cruz

Biotechnology) and Flag (F1804-1MG, Sigma). Cells were washed once in PBS and fixed for 10 minutes in 4% PFA PBS. Cells were washed and (1:1000) secondary antibodies (invitrogen) were added for 30 min. at room temperature. Monocytes were deposited on poly-lysine coated coverslips for 20 minutes at RT. Coverslips were washed twice in PBS and mounted with ProLong (Sigma).

TLR4 luciferase reporter gene assay was performed by InvivoGen (Toulouse, France).

Immunohistochemistry

MSI CRC patients were selected as previously described (Collura et al., 2014). Tumor sections from patients were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Antigens were then unmasked in pH 6,0 citrate buffer (30 min, 95°C), cooled for 30 min, washed twice in PBS for 3 min and treated with 3% H_20_2-PBS for 15 min in order to inhibit endogenous peroxidases. After washes in PBS, the slides were saturated for 25 min in 3% BSA PBS. Sections were then incubated with CD68 (1/100, M0814, Dako, Les Ulis, France), CD163 (1/100, NCL-CD163; Leica, Nanterre, France) or HSP110 (1/1200, clone 5812, Leica Biosystems) primary antibody overnight at 4°C (CD68, and CD163) or 1 h at room temperature. After washing in PBS, secondary antibody was added for one hour at room temperature. Slides were washed twice for 5 min in PBS and revealed using Novared kit (Vector, Burlingame, USA). Slides were washed twice in water for 5 minutes and counterstained with 10% Meyer's hematoxylin. After one wash in water, slides were dehydrated in 100% ethanol and in xylene for 30 sec each. The slides were then observed using the Cell Observer station (Zeiss, Germany). Positive cells were counted in three distinct stroma areas of 645µM by 482µM for each tumor in a blinded manner.

Quantitative real-time PCR.

Total RNA was isolated with Trizol (Invitrogen), reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with random hexamers (Promega). Primers for real-time PCR were from Bio-Rad (Hercules, CA, USA): Human TNFa (**qHsaCED0037461), murine TNFa**

(qMmuCED0004141), murine Arginase-1 (qMmuCID0022400), murine iNOS (qMmuCID0023087). HPRT was used as invariant control (**qMmuCED0045738 or qHsaCID0016375).**

ELISA

HSP110 concentration was determined by an ELISA. Briefly, a 96-wells plate (MaxiSorp Plate; Nunc, Sigma Aldrich, Australia) was coated in 0.2 M sodium carbonate/bicarbonate buffer, pH 9.4, overnight at 4°C with 3 µg/mL of rabbit anti-HSP110 Ab (sc-6241, Santa Cruz Biotechnology). The plates were washed and then blocked with 2% BSA in PBS for 1h at room temperature. Supernatant were diluted and added to the plates along with recombinant HSP110 produced in HEK293 cells (OriGene Technologies, Rockville, MD) to establish a standard concentration curve. The plates were then incubated 2h at room temperature, washed 3 times in PBS 0.5% Tween 20, and incubated 2h with a mouse anti-HSP110 Ab (1:200)(NCL-HSP105, Leica Biosystems). After 3 washes as previous, the plates were incubated 1h with a Goat anti-mouse IgG conjugated to alkaline phosphatase (SouthernBiotech, Birmingham, Alabama). After 3 final washes, The ELISA was developed by adding a TMB substrate reagent (OptEIA, BD Biosciences, San Jose, CA). the reaction was stoped after 30 min by addition of 2M sulfuric acid. ODs were measured at 450 nm.

Secreted HSP27 by CRC cell lines was detected using the Immunoset HSP27 high sensitivity (Human) ELISA according to the manufacturer's instructions (ADI-960-076, Enzo life sciences, Farmingdale, NY).

Statistics

Analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, Calif). The Student paired *t* test was used, where appropriate, and a 2-tailed *P* value of .05 or greater was considered significant.

Results

***In vivo*, in patients and mice, expression of HSP110 in colorectal tumors influences the profile of infiltrating macrophages.**

We first determined the presence of macrophages within tumor biopsies from CRC MSI patients, which were selected based on HSP110 expression and divided into two groups: HSP110-low (large T17 deletions, good prognosis patients group) and HSP110-high (small T17 deletions, bad prognosis group)(Collura et al., 2014) (Figure 1A). We observed a strong invasion of CD68+ macrophages in all tumor samples regardless the expression of HSP110 (Figure 1B and supp Figure 1A). However, in HSP110 high tumors, compared to HSP110 low, there was an important increase in macrophages that expressed CD163 ($p=0.0025$, $n=5$ per group), a well-described pro-tumoral (M2) macrophages marker (Figure 1B and C).

Since low expression level of HSP110 in MSI CRC cells inversely correlates with the length of the T_{17} mononucleotide repeat located in intron 8 of *HSP110,* we next used a HCT116 sub-clone displaying a large *HSP110* T17 deletion (HCT116-C22) (Collura et al., 2014) to determine the effect of HSP110 on macrophages phenotype. We first confirmed the very low expression of HSP110 in this HCT116-C22 clone compared to parental HCT116 (Figure 1D). Other than HSP110, no other HSPs expression seemed altered in HCT116-C22 (Figure 1D). Furthermore, the low expression of HSP110 in the HCT116-C22 clone did not affected HCT116 spontaneous cell death observed when cultured *in vitro* (sup figure 1B). Tumor xenografts with high or low expression of HSP110 were established by subcutaneously inoculating nude mice with HCT116 or HCT116-C22 cells, respectively. Three weeks after inoculation, xenografts were excised, and the infiltrating macrophages were examined. Immunostaining revealed in all tumors slides the presence of $F4/80^{pos}$ macrophages (figure 1E). Interestingly, whereas the macrophages from parental HCT116 xenografts expressed the M2 marker Arginase-1 (Arg-1), only the low HSP110 expressing HCT116-C22 xenograft expressed strongly the inducible *NO* synthase (iNOS), which is a marker associated with a M1 cytotoxic phenotype (Figure 1E and F). Accordingly, mice

xenografted with HCT116-C22 expressed more TNFa mRNA (Figure 1G). These data, both in human and in mice, suggest that level of HSP110 in MSI tumors may influence the profile of tumor infiltrating macrophages *in vivo*.

HSP110 is secreted by cancer colorectal cells and influences macrophage differentiation.

To decipher the mechanism of macrophage polarization observed in the presence or absence of HSP110, we studied the secretome of HCT116 and HCT116-C22. Among the 36 cytokines analyzed none were significantly modified (data not shown). In contrast, we observed the presence of a soluble HSP110 in the supernatant of HCT116 but not in HCT166-C22 (Figure 2A). The presence of extracellular HSP110 in the supernatant was confirmed by ELISA, where the amount of HSP110 secreted by different cancer colorectal cells was quantified (sup Figure 1C). We thus hypothesized that extracellular HSP110 could be involved in the macrophage infiltration and polarization observed herein. To test this, we studied the effect of supernatants of HCT116 and HCT116-C22 cells in primary human monocytes induced to differentiate into macrophages by M-CSF. As shown in figure 2B, supernatants from HCT116-C22, compared to that of HCT116, contribute to generate macrophages with a stronger expression of HLA-DR, but a lower expression of the M2 markers CD163 and CD206. Upon LPS stimulation, HCT116-C22 supernatant, compared to parental HCT116 supernatants, generated macrophages with higher levels of TNFa mRNA (supp Figure 1D), secreted higher levels of the pro-inflammatory cytokines TNFa and IL1b, and produced higher level of NO (Figure 2C). Conversely, CCL24, a chemokine associated with M2 phenotype, was significantly lower. As the capacity to stimulate T cell proliferation is a hallmark of pro-inflammatory M1 macrophages, we performed a mixed lymphocyte reaction and observed that more proliferating T cells were generated in the presence of HCT116-C22 supernatant, compared to HCT116 supernatant (Figure 2D).

To confirm that this effect on macrophage polarization was HSP110-dependent, we next down-expressed HSP110 using siRNA (supp Figure 2A). Of note, in our experimental conditions, HSP110 knockdown had no effect in the cells' viability (supp figure 2B). A lower expression of M2 markers

CD163 and CD206 was observed when using supernatants from both HSP110-depleted HCT116 (Figure 2E) and in SW480 cells (sup Figure 2C), compared to the corresponding controls. Accordingly, upon LPS stimulation, higher levels of TNFa (HCT116, Figure 2F, and SW480, supp Figure 2D) and IL1b were observed with the HSP110-depleted cells' supernatants.

HSP110DE9 mutant inhibits HSP110 release into the extracellular medium.

HSP110DE9 is a HSP110 deletion mutant, which contains only the 1-381 amino acid ATP-domain of HSP110, found in all MSI CRC patients with good prognosis (Collura et al., 2014). We have previously demonstrated that HSP110DE9 overexpression in CRC cell lines blocks HSP110 anti-aggregation and anti-apoptotic intracellular functions (Dorard et al., 2011). To study whether HSP110DE9 may also affect extracellular HSP110, we analyzed the supernatants from CRC cells overexpressing HSP110DE9 (GFP-tagged). In parallel, supernatants from the same cells overexpressing HSP110 (GFP tagged) were also tested. Interestingly, while as expected HSP110 overexpression led to an increase of its concentration in the supernatant (figure 3A), HSP110DE9 expression induced a strong decrease in the amount of HSP110 secreted, without altering the intracellular level of HSP110 (Figure 3B and C), or altering the secretome profile (supp figure 2E).

Accordingly to these variations in secreted HSP110, a stronger expression of HLA-DR and a decrease of CD206 expression were observed when HSP110DE9 was expressed (Fig 3D). Conversely, CD163 and CD206 were both significantly increased when HSP110 was overexpressed (Fig 3D). Accordingly, macrophages generated with HSP110DE9 overexpressing CRC cells' supernatants secreted higher levels of TNFa and had higher level of NO than control-GFP or HSP110 overexpressing CRC cells' supernatants (Figure 2E). Finally, more T cells were induced to proliferate in the presence of HSP110DE9 expressing cells' supernatant compared to control (Figure 3F).

All together, our results suggest that low secretion of HSP110 (either through siRNA-mediated depletion or by expressing the HSP110DE9 mutant) may favor the generation of macrophages with increased pro-inflammatory and cytotoxic functions.

Immunodepletion of HSP110 modulates the pro-inflammatory phenotype of macrophages.

We next studied if immunodepletion of extracellular HSP110 in the supernatants was enough to switch from their ability to induce anti-inflammatory macrophages to the induction of macrophages with pro-inflammatory functions. The reduction in extracellular HSP110 (but not other HSPs) after immunodepletion of HSP110 from both HCT116 and SW480 cells supernatant is shown in Figures 4A-B and supp Fig 3A. Macrophages induced to differentiate with the HSP110-immuno-depleted supernatants show a reduced expression of CD163, CD206 and increased TNFa and IL1b secretion (figure 4C and 4D for HCT116, and supp Figures 3B-C for SW480). We concluded that HSP110 might be a major molecule in the supernatants responsible of the effect observed on macrophages profile.

Extracellular HSP110 binds to TLR4 in human monocytic cells

We previously showed that HSP70 binds to and activates TLR2 on the surface of myeloid-derived suppressive cells (Chalmin et al., 2010). To study if extracellular HSP110 action on macrophages could also implicate a TLR pathway, we first determined whether its ability to induce the expression of the CD206 macrophage marker was modified in the presence of neutralizing antibodies against TLR2, TLR4 or the scavenger receptor SRA. We found that only neutralizing antibodies against TLR4 significantly reduced HSP110 containing supernatant ability to induce CD206 (Figure 5A). Accordingly, when added to the cells human recombinant HSP110 produced by eukaryotic cells (i.e. LPS free), a co-localization of this extracellular HSP110 with TLR4 was observed (Figure 5B). To confirm the implication of TLR4 in HSP110 effect on macrophages, we used TLR4 luciferase reporter cells. We incubated the TLR4-reporter or control cells with either the supernatant of SW480 cells overexpressing HSP110 (where HSP110 was measured by ELISA) or a similar amount of purified HSP110 (600 ng/mL). We found that both sources of HSP110 had a similar effect on TLR4 activation (Figure 5C). Taken together, these results suggest that extracellular HSP110 effect altering the inflammatory profile of macrophages involves the TLR4.

Discsussion

HSPs are chaperones frequently overexpressed in cancer cells. A large body of literature describes how their intracellular function is involved in the increase resistance of tumor cells to cell death notably induced by anti-cancer drugs or hypoxia (Ciocca et al., 2013; Jego et al., 2013). We recently demonstrated that HSP110 was the main HSP involved in colorectal tumorigenesis. We demonstrated that its expression was directly associated with bad patients' outcome and that the presence of an HSP110 inactivating mutation was directly associated to the good prognosis of CRC MSI patients (Dorard et al., 2011). Interestingly, in these good outcome patients, there was also an increase in the immune cells infiltrating the tumor (Banerjea et al., 2004; Boissiere-Michot et al., 2014; Deschoolmeester et al., 2010; Maby et al., 2015; Phillips et al., 2004). In this work we demonstrated that these two events associated with CRC good prognosis (ie HSP110 expression and tumor immunosurveillance) may be linked. Indeed, we show that HSP110 is secreted by CRC cells and skew the macrophages inflammatory profile. Depletion of extracellular HSP110 by the use of an antibody or by overexpressing the HSP110DE9 mutant induces the macrophages pro-inflammatory/cytotoxic potential. On the opposite, overexpression of HSP110 or addition of HSP110 recombinant protein (produced in eukaryotes to avoid LPS contamination) favors the formation of macrophages with an anti-inflammatory profile.

Although the way through which HSPs are released into the extracellular medium is still a debated issue (active versus passive secretion), it is well known that some HSPs are abundant extracellular proteins notably in the tumor microenvironment (Sherman and Multhoff, 2007). They are believed to act as DAMPs and to have immunogenic properties (Joly et al., 2010) and the term chaperokines has been advanced (Asea, 2003). Our results indicating a release of HSP110 by colorectal cancer cells is in agreement with Colgan *et al* that described that soluble HSP110 is a member of the luminal components of the non malignant human and mouse gastrointestinal tract. Immunohistochemistry showed expression of HSP110 in epithelium from the small and large intestine (Colgan et al., 2003). As

in vitro measurements showed low extracellular HSP110 concentration in MSI type CRC cells lines, it would be interesting to monitor variation of extracellular HSP110 amount within body fluids as a new biomarker of disease characterization, or progression.

The present uncovered anti-inflamatory role of the secreted form of HSP110 in CRC is in agreement with the overall literature about other extracellular HSPs. Indeed, several studies using immunizations of animals with mammalian or mycobacterial HSPs in the context of autoimmune or inflammatory diseases (Arthitis, diabetis…) have shown animal health improvement, suggesting an immunosuppressive role of HSPs (van Eden et al., 2005). This effect is probably mediated by a direct modulation/inhibition of antigen-presenting cells activation. In this way, recombinant HSP27 has been shown to directly inhibit dendritic cells differentiation and skew towards a macrophage phenotype (Laudanski et al., 2007). Furthermore, these macrophages acquire tolerogenic properties *in vitro* and in breast cancer patients (Banerjee et al., 2011). Similar immunosuppression was observed for higher molecular weight HSPs like recombinant HSP70 as Ferat-Osio et al showed that highly purified HSP70 inhibits TNFa production by monocytes (Ferat-Osorio et al., 2014). It is worth noting that the absence of minute amounts of contaminating endotoxin is mandatory to reveal the inherent suppressive functions of these HSPs as nicely demonstrated by Stocki (Stocki and Dickinson, 2012; Stocki et al., 2012). This very low level of contamination could account for the maturation effect of recombinant HSP110 on dendritic cells observed by Manjili et al. (Manjili et al., 2005). In our setting, we got rid of this caveat as we used a HSP110 naturally present in the supernatant of CRC cell lines and, when we used purified HSP110, it was produced in an eukaryotic setting with no traces of LPS.

In the absence of LPS, extracellular HSPs have been shown to bind to and activate different TLRs. In particular, we have previously shown that HSP70, expressed at the surface of tumor-derived exosomes, activated myeloid-derived suppressive cells trough its binding to TLR2 (Chalmin et al., 2010). Concerning circulating HSP27, we and others have been shown that it inhibits macrophages and dendritic cells differentiation through TLR4 (Laudanski et al., 2007) and it favors angiogenesis through

TLR3 (Thuringer et al., 2013). We show here that soluble HSP110's anti-inflammatory effect on macrophages involves TLR4. kuang et al have shown that TLR4 signaling on monocytes could be deleterious for their adequate activation and differentiation (Kuang et al., 2007). They demonstrated that hyaluronan secreted from tumors binds TLR4 on monocytes that become refractory to subsequent stimulation. In addition, depending on the context, NFkB signaling induced upon TLR2/4 can transmit an anti-inflammatory message in macrophages (Fong et al., 2008; Greten et al., 2007; Mancino and Lawrence, 2010). Finally, IRAK-M, a negative regulator of TLR signaling that can be upregulated in tumor-infiltrating macrophages in a TLR4-dependent manner, could interfere with an adequate polarization (del Fresno et al., 2005). Taken together, these mechanisms may provide a rational for the effect observed with HSP110. It is therefore possible that the early presence of HSP110 during the monocytes differentiation process could hamper the subsequent pro-inflammatory function of macrophages, as a signal of a refractory state.

Other HSP-binding receptors have been described over the last few years. Among them, scavenger receptor SRA/CD204 is a described receptor for high molecular weight HSPs such as HSP110 (Facciponte et al., 2007; Qian et al., 2011). Although in our experimental setting this receptor was not involved, other authors have shown that it mediates an inhibitory signal in dendritic cells upon HSP110 stimulation, supporting our hypothesis that extracellular HSP110 is deleterious to optimal immune responses. Thus, as suggested by W. Van Eden for other HSP family members, HSP110 should therefore not be considered as a DAMP (accordingly to the concept of "danger" introduced by P. Matzinger) but rather as a "DAMPer" of the immune system (Broere et al., 2011).

Several groups have investigated the nature of the immune infiltrate within tumors of CRC patients. Though all groups agree on the higher Th1 cells colonization, they provide puzzling observations regarding myeloid cells. The presence of CD163+ macrophages in the tumor microenvironment has been described in MSS and MSI CRC biopsies (Forssell et al., 2007; Llosa et al., 2015). Surprisingly, concomitant increases in M1 and M2 subsets were associated with a better prognosis (Edin et al.,

2012). However, a high expression of PD-L1, an immune-inhibitory ligand, at the surface of myeloid cells was found at the invasive front and in the stroma of CRC MSI biopsies, suggesting that these cells provide a negative signal to T cells, thus dampening the immune response (Le et al., 2015; Llosa et al., 2015). Macrophages become, therefore, a cellular target through PD-1/PD-L1 blockage. Such a strategy is currently investigated in various tumors with mismatch-repair deficiency, including CRC, with encouraging results (Le et al., 2015).

Studies on immune tumor microenvironment distinguished CRC patients only on a microsatellite stability basis (i.e. MSS versus MSI). However, we have recently shown that HSP110 expression is a strong and reliable marker to distinguish good from bad prognosis within MSI patients. In terms of patients' outcome, high HSP110 expressing MSI patients are likely to be not distinguishable from MSS patients. Here we show that these patients with high HSP110 have a greater invasion of CD163+ macrophages than the good prognosis MSI patients with low HSP110 expression. Based on these results, we have started a clinical study to determine if a correlation exists between CD163+ macrophages, HSP110 and survival. It remains also to be established whether an association exist between CD163+ cells and lymphocytes infiltration, as this last is a well established predictor of overall survival and relapse in CRC (Galon et al., 2006).

Over the recent last years, HSP110 has become a new point of interest in the field of HSP and cancer, mainly thanks to its excellent chaperoning capacity of peptide antigens that makes it a powerful tool for vaccines development (Mattoo et al., 2013; Wang and Subjeck, 2013). Until our recent published works demonstrating the essential role of HSP110 in CRC, its role in tumor development remained almost unexplored. This study brings new information to the emerging role of extracellular HSP110 in the inhibition of the immune system in the context of tumor microenvironment. It confirms the necessity to target extracellular in addition to intracellular HSP110 in CRC and should foster the development of specific inhibitors that are strongly lacking for this HSP.

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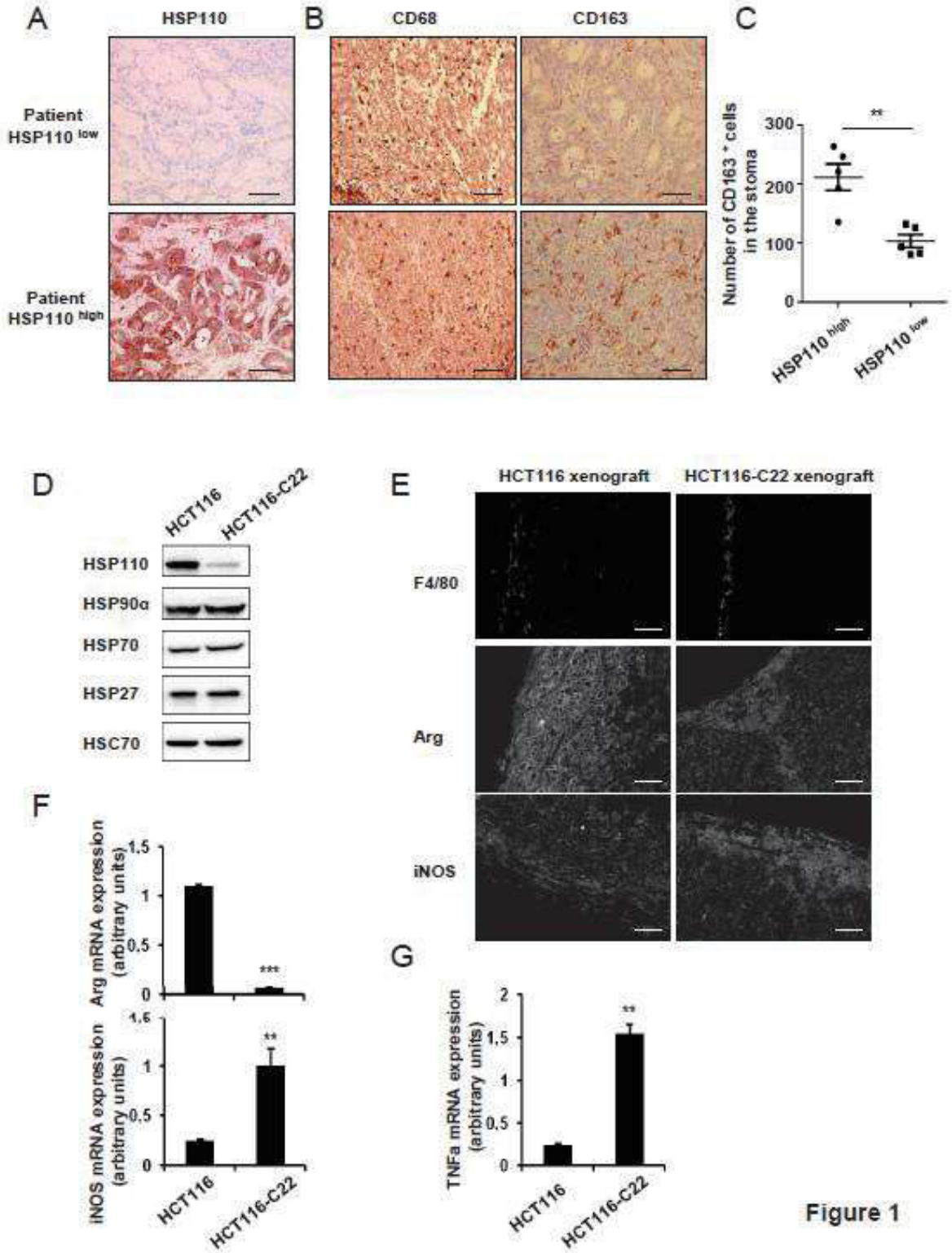
A
HSP110
Patient HSP110 low
Patient HSP110 high
B
CD68
CD163
C
Number of CD163 + cells in the stoma
300
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HSP110 high
HSP110 low
D
HCT116
HCT116-C22
HSP110
HSP90α
HSP70
HSP27
HSC70
E
HCT116 xenograft
HCT116-C22 xenograft
F4/80
Arg
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Arg mRNA expression (arbitrary units)
1,5
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iNOS mRNA expression (arbitrary units)
1,5
1
0,5
0
**
HCT116
HCT116-C22
G
TNFa mRNA expression (arbitrary units)
2
1,5
1
0,5
0
**
HCT116
HCT116-C22

Figure 1

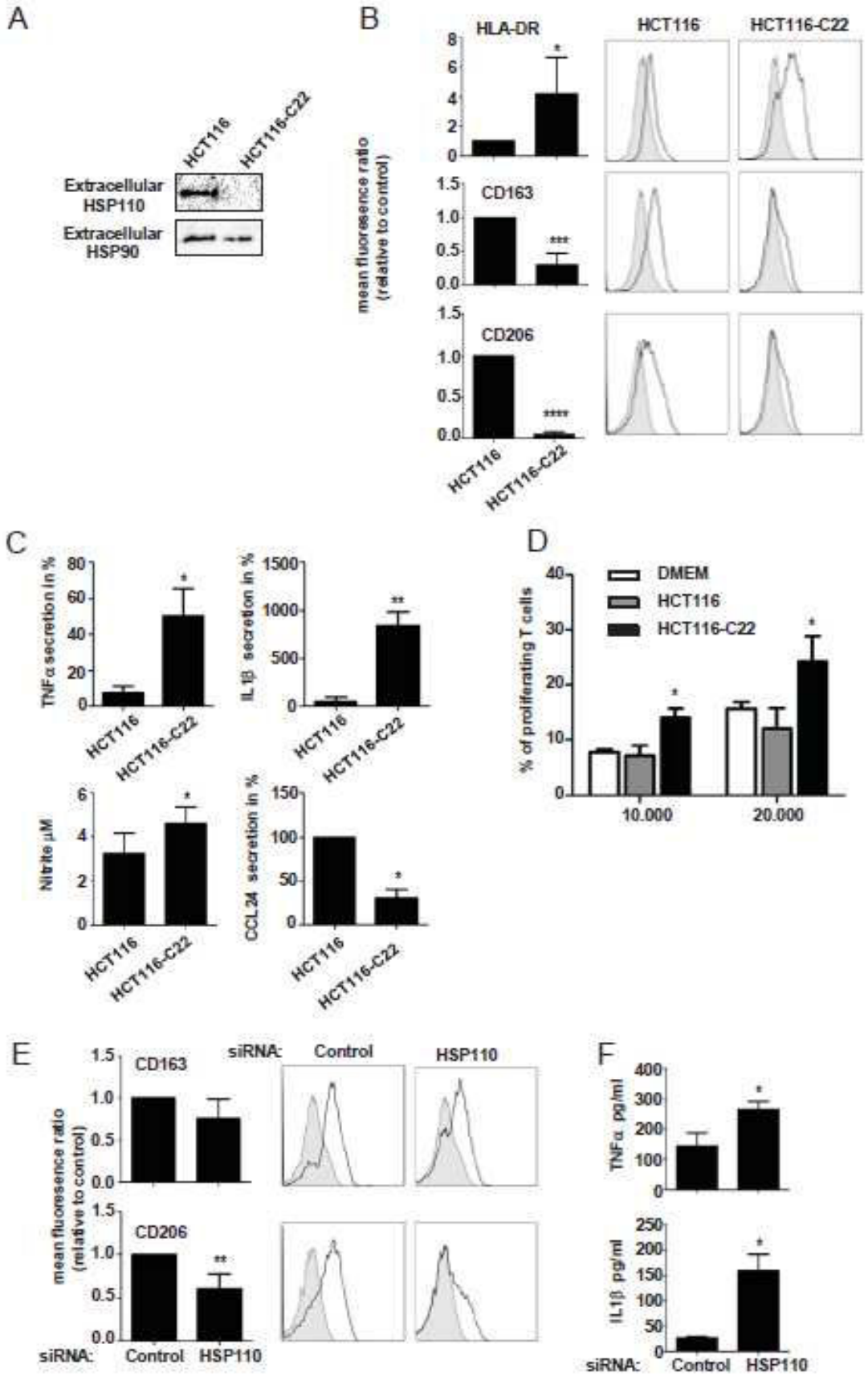

A
HCT116
HCT116-C22
Extracellular HSP110
Extracellular HSP90
B
HLA-DR
HCT116
HCT116-C22
mean fluorescence ratio (relative to control)
CD163
CD206
HCT116
HCT116-C22
C
TNFα secretion in %
IL1β secretion in %
Nitrite µM
CCL24 secretion in %
HCT116
HCT116-C22
D
DMEM
HCT116
HCT116-C22
% of proliferating T cells
10.000
20.000
E
siRNA: Control HSP110
CD163
CD206
mean fluorescence ratio (relative to control)
siRNA: Control HSP110
F
TNFα pg/ml
IL1β pg/ml
siRNA: Control HSP110

Figure 2

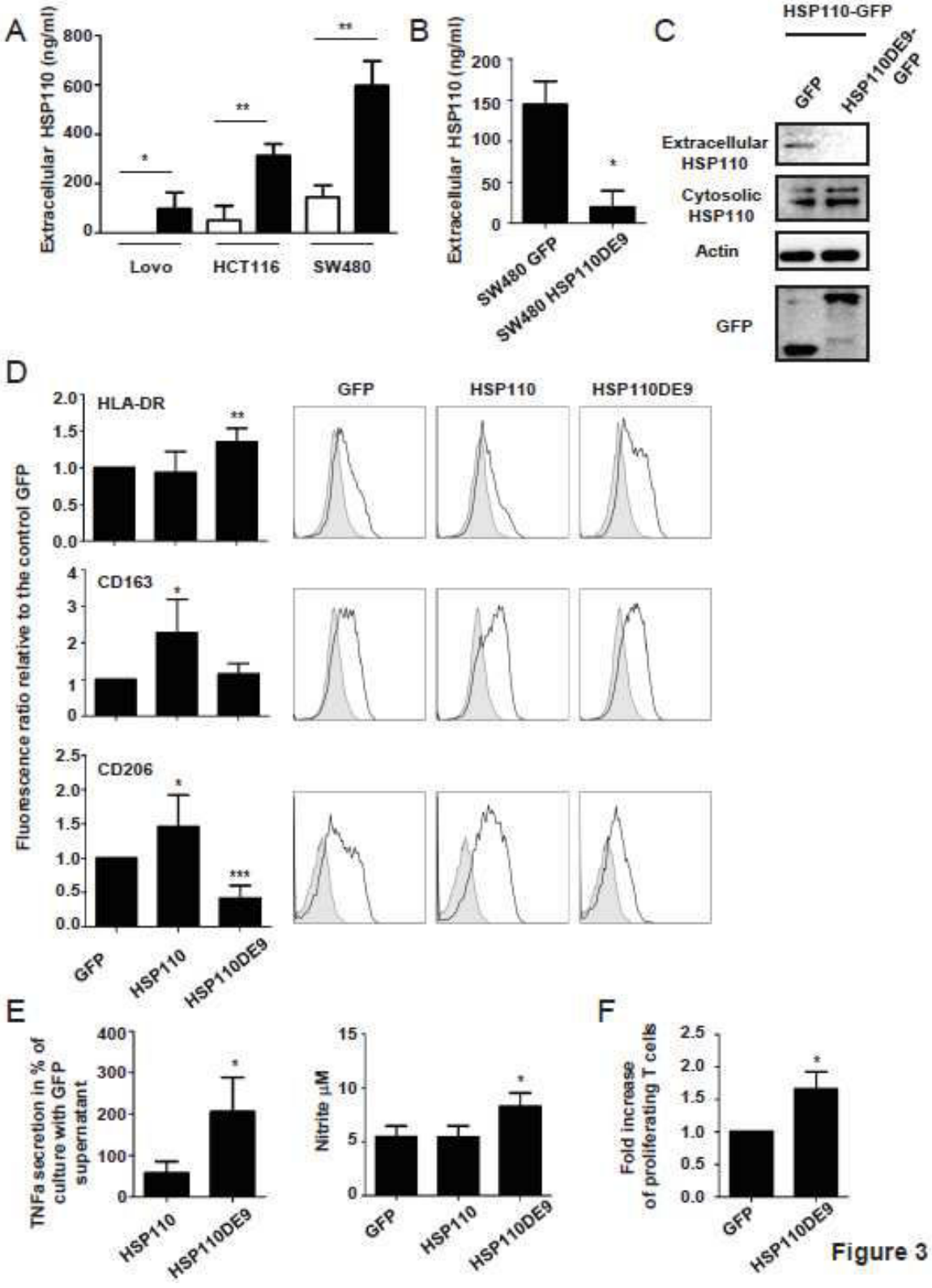

A
Extracellular HSP110 (ng/ml)
800
600
400
200
0
*
**
**
Lovo
HCT116
SW480
B
Extracellular HSP110 (ng/ml)
200
150
100
50
0
*
SW480 GFP
SW480 HSP110DE9
C
HSP110-GFP
GFP
HSP110DE9-GFP
Extracellular HSP110
Cytosolic HSP110
Actin
GFP
D
GFP
HSP110
HSP110DE9
HLA-DR
CD163
CD206
Fluorescence ratio relative to the control GFP
GFP
HSP110
HSP110DE9
E
TNFa secretion in % of culture with GFP supernatant
HSP110
HSP110DE9
Nitrite µM
GFP
HSP110
HSP110DE9
F
Fold increase of proliferating T cells
GFP
HSP110DE9

Figure 3

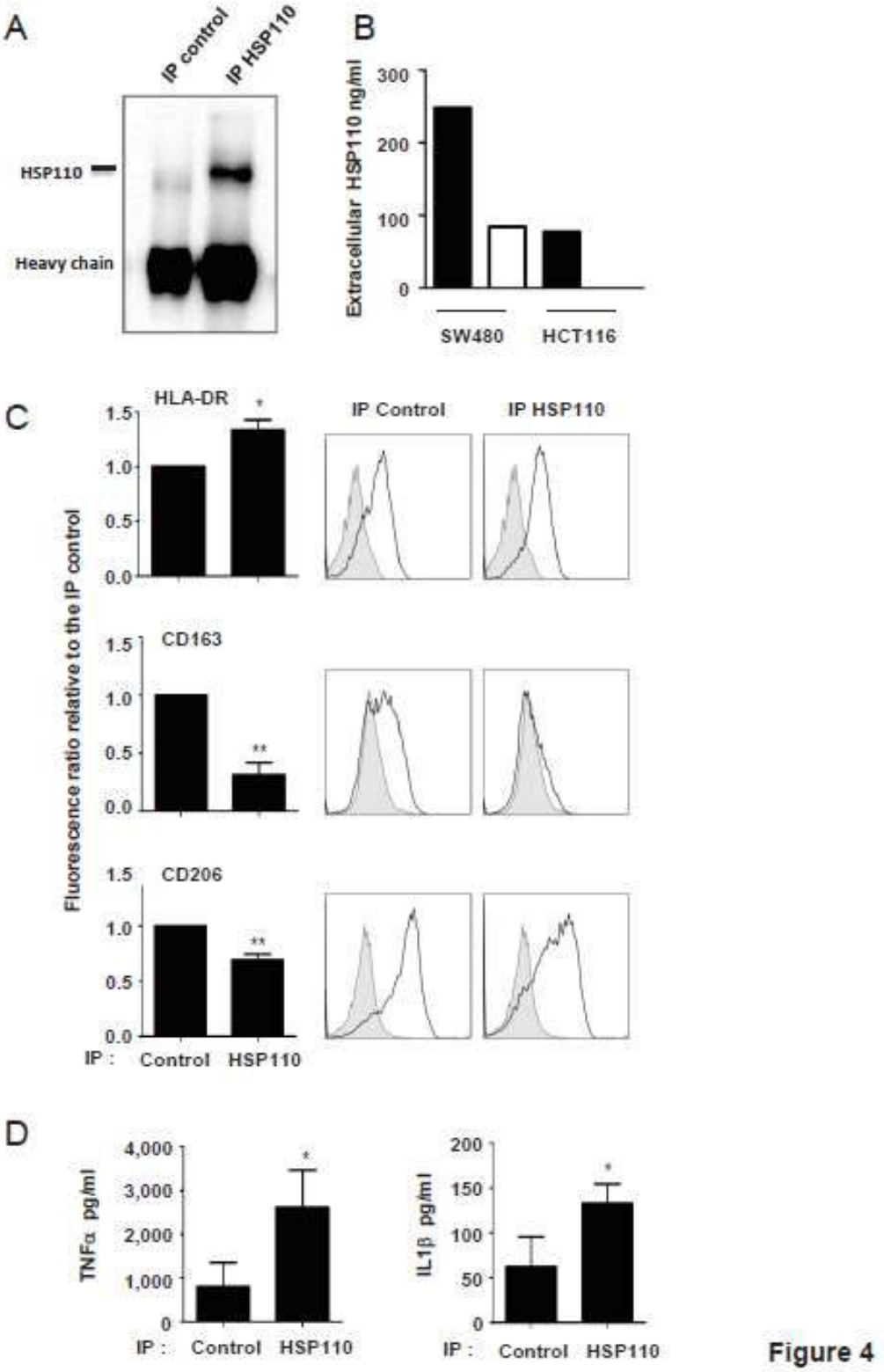
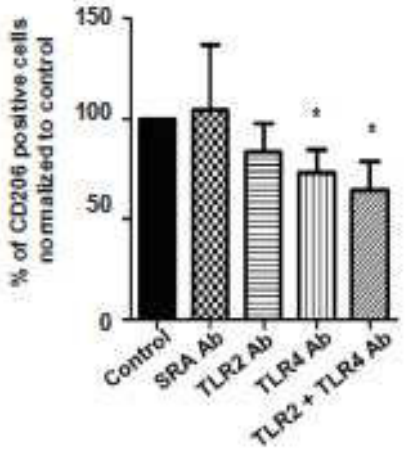
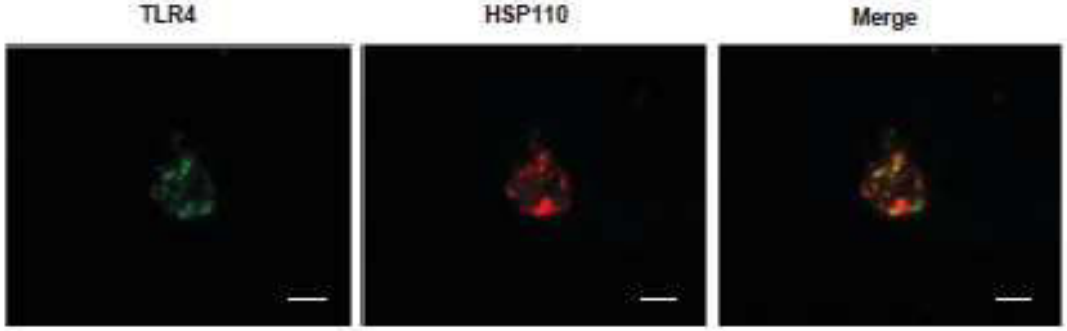

A
IP control
IP HSP110
HSP110
Heavy chain
B
Extracellular HSP110 ng/ml
300
200
100
0
SW480
HCT116
C
HLA-DR
IP Control
IP HSP110
CD163
CD206
Fluorescence ratio relative to the IP control
1.5
1.0
0.5
0.0
IP : Control HSP110
D
TNFα pg/ml
4,000
3,000
2,000
1,000
0
IL1β pg/ml
200
150
100
50
IP : Control HSP110

Figure 4

A


% of CD206 positive cells normalized to control
150
100
50
0
*
*
Control
SRA Ab
TLR2 Ab
TLR4 Ab
TLR2 + TLR4 Ab

B


TLR4
HSP110
Merge

C

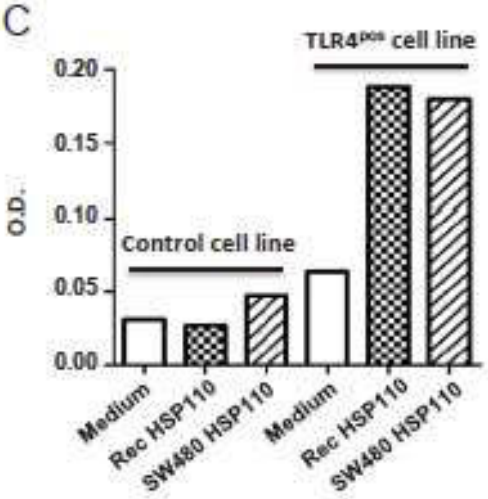

TLR4pos cell line
0.20
0.15
0.10
0.05
0.00
O.D.
Control cell line
Medium
Rec HSP110
SW480 HSP110
Medium
Rec HSP110
SW480 HSP110

Figure 5

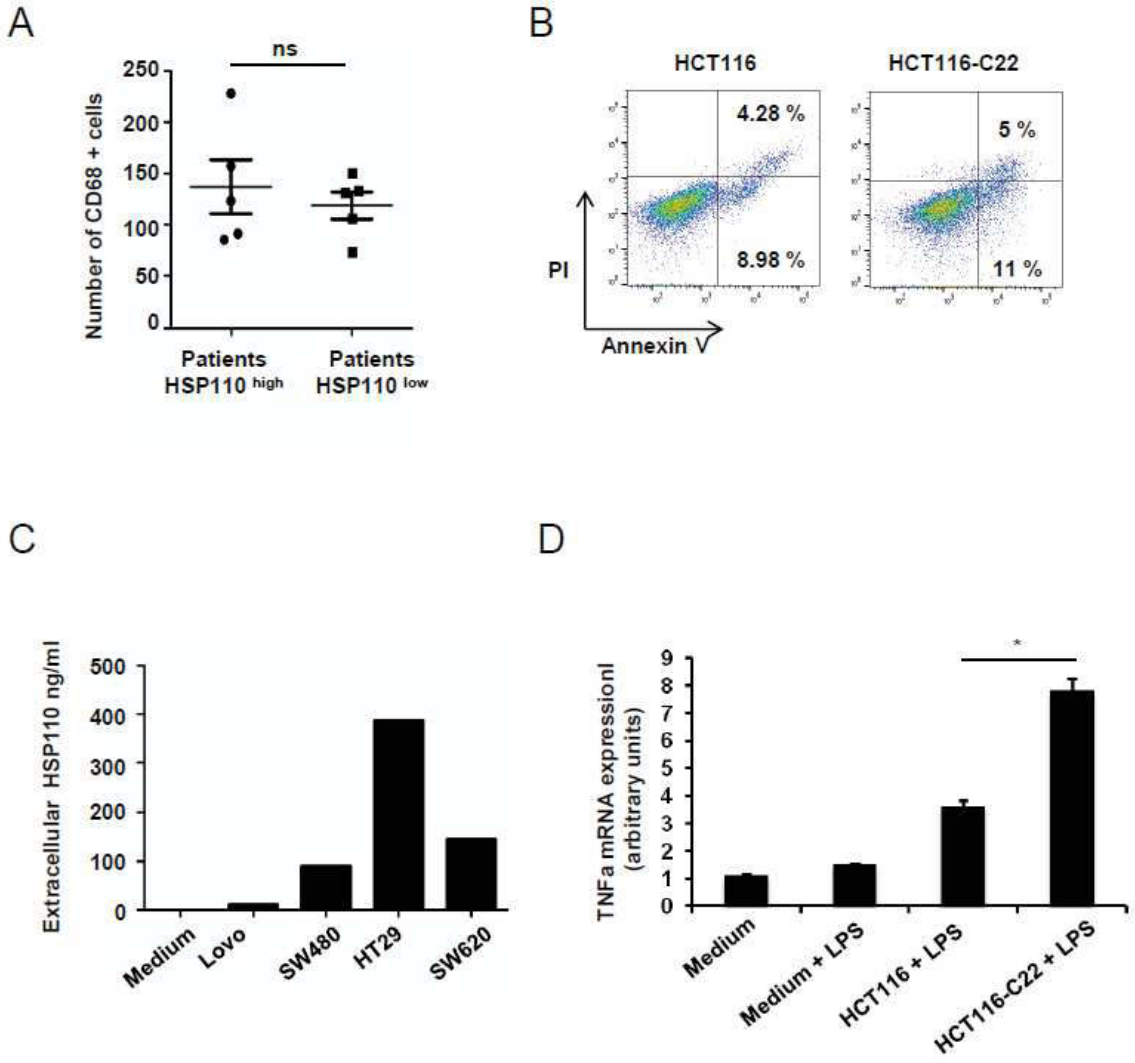
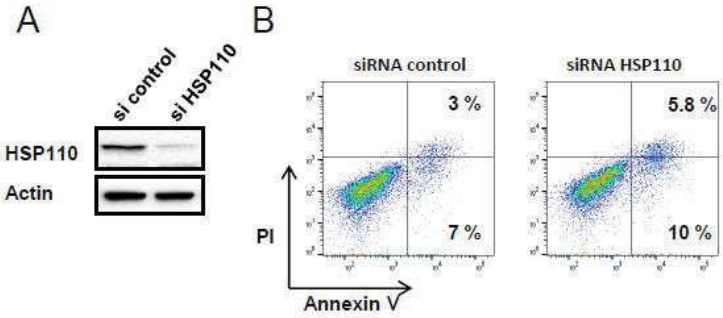
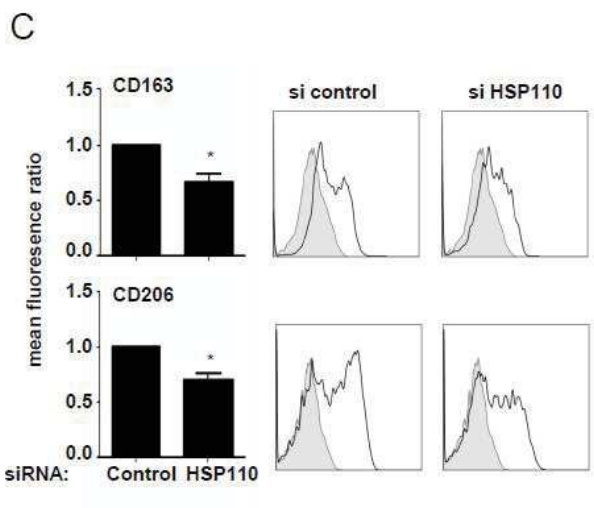
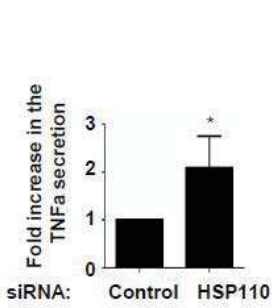
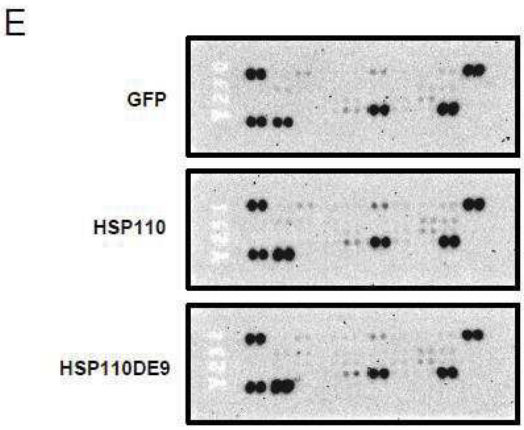


Figure supp 1









C+	C5a	CD40L	G-CSF	GM-CSF	GROa	I-309	sICAM-1	IFNg	C+
	IL-1a	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
	IL-10	IL-12p70	IL-15	IL-16	IL-17	IL-17e	IL-23	IL-27	
	IL-32a	IP-10	I-TAC	MCP-1	MIF	MIP-1a	MIP-1b	Serpin E1	
C+	RANTES	SDF-1	TNF	sTREM					C-

Figure supp 2

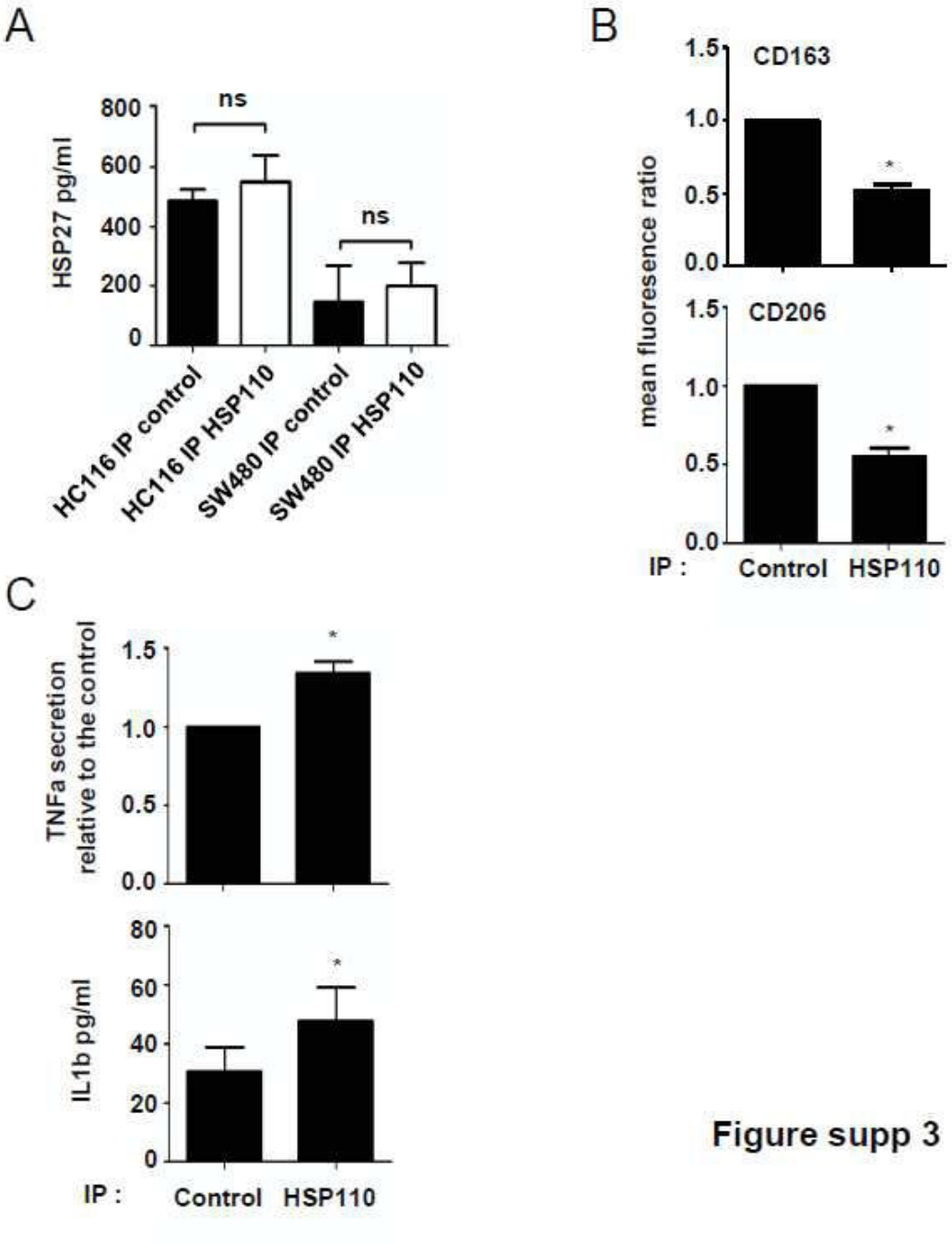


Figure supp 3

Figures Legends.

Figure 1. Pro-tumoral macrophages invade tumor bed in HSP110 ^{high} expressing CRC

A, B Expression of HSP110 (A), CD68 and CD163 (B) by IHC in tumor samples from MSI CRC patients belonging to the HSP110 ^{high} and HSP110 ^{low} groups described by Collura et al (reference 4). One representative image is shown (n=5) Brown color indicates positive staining (200x magnification for A, scale bars, 50 μm. 100x magnification for B, scale bars, 100 μm). C) Number of CD163 macrophages in tumors biopsy stroma was determined (n=5)(value for each patient was determined as the average number of stained cells in 3 distinct sections. D) Indicated HSPs were analyzed by Immunoblot in HCT116 and HCT116-C22 cells. HSC70 was used as a loading control. E) Expression of F4/80, iNOS and Arginase by IHC from tumor sections of mice xenografted with HCT116 or HCT116-C22 (One image representative of 6 mice in each group, 20x magnification, scale bars 40 μm). (F, G) qPCR analysis of Arginase, iNOS mRNA (F) and TNFa (G) from tumor sections of mice xenografted with HCT116 or HCT116-C22. **, $p<0.01$, ***, $p<0.005$

Figure 2. **HSP110 is secreted by CRC cell lines and influences macrophage differentiation profile.**

A) Immunoblot analysis of HSP110 in the supernatant of HCT116 and HCT1166-C22. Extracellular HSP90 is used here as a loading control. B) Differentiating macrophages in the presence of HCT116 or HCT116-C22 supernatants were assessed for the expression of HLA-DR (n=7), CD163 (n=5), and CD206 (n=5) by flow cytometry. Results are expressed as mean fluorescence ratio *, $p<0.05$; ***, $p<0.005$; Left, data of all experiments; Right, representative data. C) Monocytes induced to differentiate in the presence of HCT116 or HCT116-C22 supernatants were stimulated for 24h with LPS. TNFa (n=3), IL1b (n=3), CCL24 (n=4) and NO (n=4) secreted by macrophages were determined by Milliplex assay *, $p<0.05$; **, $p<0.01$. D) Percentage of proliferating allogeneic T cells after 3 days of culture with macrophages derived from monocytes in the presence of control DMEN medium or supernatants from HCT116 or HCT116-C22 cells (n=3), *, $p<0.05$. E, F) Monocytes were induced to differentiate into macrophages in the presence of supernatant from HCT116 cells either transfected with a HSP110

siRNA or a scrambled control. Expresssion of CD163 (n=5), and CD206 (n=5) was determined by flow cytometry (E). F, TNFa and IL1b from macrophages derived from monocytes in the presence of supernatants as in E, and stimulated for 24h with LPS. (n=3) *, p<0.05 ; **, p<0.01

Figure 3. HSP110DE9 hampers HSP110 release

A) Concentration of extracellular HSP110 in the supernatant of Lovo, HCT116 and SW480 transfected with a control GFP plasmid or a plasmid coding HSP110-GFP and measured by ELISA (n=3) *, p<0.05 ; **, p<0.01 . B) ELISA quantification of HSP110 in the extracellular medium of SW480 transfected with a control GFP or HSP110DE9-GFP plasmid. (n=3) *, p<0.05 . C) Immunoblot analysis of HSP110 in the supernatant of HCT116, transfected with a HSP110-GFP plasmid with or without a plasmid coding HSP110DE9-GFP. D) Flow cytometry analysis of HLA-DR (n=6), CD163 (n=6), and CD206 (n=6) expression on macrophages derived from monocytes in the presence of supernatant from SW480 cells transfected with a control GFP, HSP110-GFP or a HSP110DE9-GFP plasmid . Left, data of all experiments; Right, representative data *, p<0.05 ; **, p<0.01; ***, p<0.001. E), TNFa, and NO (nitrite) secreted by macrophages derived from monocytes in the presence of SW480 transfected as in C, and stimulated for 24h with LPS (n=4, *, p<0.05). F) Percentage of proliferating allogeneic T cells after 3 days of culture with 10^4 macrophages derived from monocytes in the presence of supernatant from SW480 transfected with a control GFP or a HSP110DE9-GFP (n=3). *, p<0.05.

Figure 4. Depletion of HSP110 from the supernatants skews the pro-inflammatory phenotype of macrophages.

A) Immunoblot analysis of HSP110 from the immunoprecipitated (IP) fraction of HCT116 supernatant (one image representative of three). B) ELISA determination of HSP110 amount in the supernatant of SW480 or HCT116 before (black columns) and after (white columns) HSP110 immunoprecipitation. C), Expresssion of CD163 (n=4), and CD206 (n=4) by flow cytometry on macrophages derived from monocytes in the presence of HSP110-depleted HCT116 supernatant. Left, data of all experiments; Right, representative data *, p<0.05 ; **, p<0.01. D, TNFa, and IL1b secreted by macrophages derived

from monocytes in the presence of HSP110-depleted HCT116 supernatant, and stimulated for 24h with LPS (n=4) *, p<0.05.

Figure 5. Extracellular effect of HSP110 on macrophages profile involves TLR4.

A) The percentage of HSP110-induced CD206-expressing differentiated macrophages in the presence or absence of SRA, TLR2 and/or TLR4 neutralizing antibodies was determined by flow cytometry. Data are expressed as percentage of the control (no neutralizing Ab added)(n=6, *, p<0.05.). B) Fluorescence microscopy analysis of TLR4 and DDK(FLAG)-tagged HSP110 purified from eukaryotic cells (LPS free) on monocytes after 30 min. of incubation with HSP110. One representative image is shown. Scale bars 10 μm. C) TLR4 gene reporter assay (luciferase) using control HSP110-depleted supernatant, supernatant from HSP110-overexpressing HCT116 cells or an HSP110 similar amount (600 ng/ml) of purified recombinant LPS-free HSP110 (n=2). *, p<0.05.

Supplementary Figures Legends.

Supp Figure 1: A) absolute count of CD68 positive cells in tumor biopsies from MSI patients (n=5) (value for each patient was determined as the average number of stained cells in 3 distinct sections), ns= non statistically significant. B) Annexin-V/IP flow cytometry analysis of HCT116 and HCT116-C22. C) Concentration of extracellular HSP110 in the supernatant of several CRC cell lines (mean of duplicate). D) qPCR analysis of TNFa mRNA from macrophages derived from monocytes in the presence of HCT116 or HCT116-C22 supernatants, and stimulated for 24h with LPS, *, p<0.05.

Supp Figure 2: A) Immunoblot analysis of HSP110 in HCT116 transfected with a control siRNA or a HSP110 siRNA. B, Annexin-V/IP flow cytometry analysis of HCT116 3 days after transfection with a control siRNA or a HSP110 siRNA. C) Expresssion of CD163 (n=3) and CD206 (n=3) by flow cytometry on macrophages derived from monocytes in the presence of supernatant from SW480 transfected with a control siRNA or a HSP110 siRNA. Left, data of all experiments; Right, representative data. *, p<0.05. D) TNFa secreted by macrophages derived from monocytes in the presence of SW480 transfected as

in C, and stimulated for 24h with LPS (n=5, *, p<0.05.). E) Secretome array of supernatant from SW480 transfected with control GFP, HSP110-GFP or HSP110DE9-GFP.

Supp Figure 3: A) ELISA quantification of HSP27 in the supernatant of SW480 or HCT116 before (black columns) and after (white columns) immunoprecipitation. ns= non statistically significant. B) Expresssion of CD206 and CD163 (n=3) by flow cytometry on macrophages derived from monocytes in the presence of HSP110-depleted SW480 supernatant, *, p<0.05. C) TNFa and IL1b secreted by macrophages derived from monocytes in the presence of HSP110-depleted SW480 supernatant, and stimulated for 24h with LPS (n=3) *, p<0.05.

Discussion générale et Perspectives

Les HSP sont des protéines chaperonnes impliquées dans le repliement des protéines nouvellement synthétisées ou dénaturées. Elles participent à la survie des cellules en condition de stress grâce à leur propriétés anti-apoptotiques et anti-agrégations. Elles sont également impliquées dans différents processus, tels que le transport de protéines à travers les compartiments cellulaires ou la différenciation cellulaire. La protéine HSP110 joue un rôle clef dans l'homéostasie de la muqueuse intestinale. La signalisation induite par le récepteur CD1d présent sur les cellules épithéliales de l'intestin entraîne l'activation de la voie STAT3 et la transcription des gènes codant pour CD1d, HSP110 et l'IL-10, cette dernière permettant ainsi de protéger les cellules de l'inflammation. HSP110 peut alors favoriser la phosphorylation de STAT3 à deux niveaux, en interagissant avec celui-ci dans la cellule, ou en activant un récepteur membranaire suite à sa sécrétion dans le lumen de l'intestin. Le mécanisme de sécrétion, tout comme le récepteur interagissant avec l'HSP110 extracellulaire dans ce contexte, n'ont pas encore été identifié. HSP110 fait donc partie d'une boucle de signalisation finement régulée, notamment par les protéines inhibitrices de l'activation de la voie STAT3. Toute dérégulation dans cette boucle peut conduire à des maladies inflammatoires de l'intestin ou contribuer au développement du cancer.

La protéine HSP110 est ainsi surexprimée dans le cancer colorectal où elle est associée à la formation de métastases et à un mauvais pronostic. Ses fonctions tumorigéniques sont cependant mal connues. L'expression d'un mutant d'HSP110, nommé HSP110ΔE9, a été mise en évidence dans les cancers colorectaux de type MSI. Ce mutant agit dans les cellules cancéreuses comme un dominant négatif, en se liant à HSP110 et en inhibant ses fonctions. L'expression de ce mutant sensibilise les cellules à la chimiothérapie et est associée à un meilleur pronostic pour les patients.

Dans ce contexte, nous avons déterminé le rôle que pourrait jouer HSP110 dans le cancer colorectal sur la régulation de différentes voies oncogéniques, et notamment de la voie STAT3. Nous avons ensuite déterminé l'effet de la sécrétion d'HSP110 par les cellules cancéreuses sur la polarisation des macrophages.

HSP110 favorise la phosphorylation de STAT3 dans les cellules cancéreuses colorectales.

L'activation de la voie STAT3 est associée à un mauvais pronostic dans les cancers. Celle-ci induit en effet la transcription de gènes impliqués dans la prolifération et la survie des cellules. La surexpression d'HSP110 contribue au développement du cancer colorectal en favorisant sa prolifération à travers l'activation de la voie STAT3. L'inhibition fonctionnelle d'HSP110, par l'expression du mutant HSP110ΔE9, inhibe à la fois la prolifération et l'activation de la voie. L'activation de la voie STAT3 induite par l'IL-6 est ainsi favorisée par l'expression d'HSP110, et inhibée par celle d'HSP110ΔE9. Ces résultats sont confirmés dans différents modèles de xénogreffe de souris et chez les patients. L'activation de la voie STAT3 dans les tumeurs MSI, tout comme la présence d'HSP110, a donc également une valeur pronostique.

La voie STAT3 n'est pas la seule voie oncogénique affectée par HSP110 dans le cancer colorectal. HSP110 favorise également l'activation de la voie β-caténine. HSP110 peut ainsi recruter la protéine phosphatase 2A au sein du complexe de dégradation de la β-caténine afin d'éviter son hyperphosphorylation, et donc son ubiquitination et sa dégradation par le protéasome. La diminution de l'expression d'HSP110 et l'expression d'HSP110ΔE9, retrouvées dans tous les cancers colorectaux de type MSI, suite à la diminution de la taille du microsatellite T17 de l'intron 8, semblent donc avoir un effet anti-oncogénique. Cette mutation ne devrait donc en théorie pas être sélectionnée. Une possibilité permettant d'expliquer la présence de cette mutation est la grande taille de la répétition (T17) dans l'intron et constituerait alors un endroit critique pour les insertions et les délétions dans les tumeurs MSI. La dégradation des ARNm codant pour HSP110ΔE9 par le système NMD pourrait alors protéger les cellules cancéreuses de l'expression délétère du mutant (Lagrange et al.; submitted). Il est également envisageable que la diminution de l'expression d'HSP110 et l'expression du mutant HSP110ΔE9 puissent présenter des avantages dans les stades précoces de la tumorigenèse du cancer colorectal MSI. En effet, bien que les protéines clientes d'HSP110 soient peu décrites, on retrouve parmi celle-ci la protéine p53. Celle-ci est notamment impliquée dans la surveillance des dommages à l'ADN et peut entraîner l'arrêt du cycle cellulaire ou l'apoptose si ceux-ci sont trop importants. La déstabilisation de p53 pourrait ainsi favoriser le développement tumoral. L'expression de p53 dans les tumeurs MSI exprimant une forte ou faible expression d'HSP110 devra être déterminée. La diminution de l'expression d'HSP110 et l'expression du mutant HSP110ΔE9 limiterait cependant ensuite les capacités invasives et métastatiques de la tumeur.

La promotion de la phosphorylation de STAT3 par HSP110 pourrait également jouer un rôle clef dans différents organes en condition normale. HSP110 est ainsi retrouvée surexprimée dans les gonades et le cerveau, et pourrait donc jouer des rôles spécifiques à ces organes. Le facteur de transcription STAT3 est ainsi impliqué dans la spermatogenèse, la plasticité des neurones et la maintenance des axones à travers la signalisation induite par le CNTF (jablonka 2014).

La sécrétion d'HSP110 par les cellules cancéreuses colorectales oriente les macrophages vers un phénotype anti-inflammatoire.

L'interaction des cellules cancéreuses avec le système immunitaire joue un rôle important dans leur développement. Les cancers colorectaux de type MSI, comparativement aux MSS, montrent une plus forte infiltration de cellules immunitaires, notamment de cellules T CD8+ et Th1, et sont associés à un meilleur pronostic. L'infiltration de macrophages dans le cancer colorectal, contrairement à tous les autres types de cancer, est également associée à un meilleur pronostic. Les macrophages sont ici polarisés vers un phénotype mixte M1/M2, les fonctions M1 semblent donc prédominer.

La sécrétion de différents facteurs, non identifiés, par les cellules cancéreuses colorectales, peut influencer la polarisation des macrophages. Les protéines HSP sont fréquemment retrouvées dans le milieu extracellulaire de l'environnement tumoral et ont généralement un effet anti-inflammatoire. Ainsi, l'immunisation d'animaux avec des HSP dans le contexte de maladies inflammatoires entraîne une amélioration de leur santé. Les HSP agissent en inhibant l'activation des CPA. HSP27 peut ainsi inhiber la différenciation des cellules dendritiques et polariser les macrophages vers un phénotype tolerogenique. HSP70 peut, quant à elle, inhiber la production de TNFα par les monocytes. Le rôle anti-inflammatoire de la forme sécrétée d'HSP110 dans le cancer colorectal est donc en accord avec la littérature. L'absence complète de LPS est cependant nécessaire pour observer l'effet immunosuppresseur des HSP. Sa présence pourrait ainsi être impliquée dans la maturation des cellules dendritiques induite par l'HSP110 purifiée ici (Manjili et al.). La protéine HSP110 ne comporte cependant pas de séquence de sécrétion. Trois mécanismes de relargage des HSP ont à ce jour été décrit. Les HSP peuvent tout d'abord être libérée suite à la mort cellulaire par nécrose, à travers le relargage d'exosomes ou par des sécrétions de vésicules lysosomales. HSP110 est cependant absente des exosomes. Le dernier mécanisme est donc le plus plausible. La sécrétion d'HSP110

semble plus faible dans les cancers colorectaux de type MSI. Il serait donc intéressant d'évaluer la quantité d'HSP110 dans le sang et l'urine comme un nouveau biomarqueur de la progression tumoral. L'effet de l'HSP110 recombinante que nous avons utilisée sur la polarisation des macrophages, bien que purifiée à partir de cellules de mammifères, est en revanche assez faible. Il est possible que l'effet médié par HSP110 soit dépendant des protéines avec lesquelles elle interagit. L'interaction d'HSP110 avec des antigènes de tumeurs permettrait l'activation du système immunitaire, alors que son interaction avec des protéines présentes dans les cellules cancéreuses auraient un effet globalement anti-inflammatoire.

Les HSP peuvent lier et activer différents TLR sur les cellules myéloïdes. L'HSP70 exprimée à la surface des exosomes sécrétée par les cellules cancéreuses peut activer les MDSC à travers leur récepteur TLR2. HSP27 peut quant à elle inhiber la différenciation des macrophages et des cellules dendritiques à travers leur récepteur TLR4, et favoriser l'angiogenèse à travers le récepteur TLR3 des cellules endothéliales. Le blocage de différents récepteurs sur les monocytes montre que l'effet sur la polarisation médié par HSP110 implique le récepteur TLR4. La signalisation TLR4 peut ainsi avoir un effet délétère sur l'activation et la différenciation des monocytes. Il est donc possible que la présence d'HSP110 empêche l'acquisition de leurs fonctions pro-inflammatoires. Le blocage du récepteur TLR4 n'est cependant pas suffisant pour bloquer totalement la polarisation médié par HSP110 et pourrait donc faire intervenir d'autres récepteurs. Le récepteur scavenger SR-A a, par exemple, été décrit comme un récepteur aux HSP de haut poids moléculaire, et notamment d'HSP110. Son activation est de plus anti inflammatoire. Ce récepteur ne semblait cependant pas impliqué dans notre modèle. La détermination des voies de signalisation activées suite à la stimulation des monocytes par des surnageants avec un fort ou faible niveau d'HSP110 permettra de mieux comprendre son effet.

Les antigènes de tumeurs peuvent être présentés par les CPA aux lymphocytes T pour initier une réponse immunitaire spécifique. Il serait intéressant de déterminer si HSP110ΔE9 peut également être présenté comme un antigène de tumeur, tout comme pour les autres protéines tronquées présentes dans les cancers colorectaux de type MSI. Il serait également intéressant de déterminer si l'expression d'HSP110, par ses propriétés chaperons, peut modifier le profil d'expression des antigènes de tumeur dans les cellules cancéreuses.

Conclusion générale

L'ensemble de ces travaux montre le rôle important d'HSP110 dans le développement du cancer colorectal. HSP110 peut ainsi favoriser la prolifération, la survie et la formation de métastases à travers l'activation de voies de signalisation oncogéniques, mais également inhiber l'activation du système immunitaire à travers sa sécrétion. HSP110 apparait donc comme une cible potentielle pour le traitement du cancer colorectal. Les cancers colorectaux MSI exprimant faiblement HSP110 montrent ainsi une meilleure réponse à la chimiothérapie. La réponse à la chimiothérapie peut être influencée par la nature de l'infiltrat de cellules immunitaires. Il serait donc intéressant dans ce contexte de déterminer si HSP110 peut jouer un rôle sur cet infiltrat. La présence d'HSP110 pourrait par exemple inhiber la mort immunogénique des cellules cancéreuses suite à la chimiothérapie, réduisant ainsi l'activation du système immunitaire. Il serait également intéressant d'évaluer le rôle d'HSP110 dans d'autres types de cancer. Les cancers gastriques présentent un très mauvais pronostic et peuvent également présenter une instabilité microsatellitaire. La détermination de la taille de la délétion du microsatellite T17 dans ces cancers pourrait avoir un rôle pronostic et permettre de sélectionner les patients qui bénéficieraient le plus de la chimiothérapie. (Zitvogel 2011 nat rev clin oncol)

L'utilisation du mutant HSP110ΔE9 pour le traitement des patients atteints de cancer colorectal présente cependant trop d'inconvénients, notamment en raison de sa grosse masse moléculaire. Le développement d'inhibiteurs chimiques, ciblant à la fois les fonctions intracellulaires et extracellulaires d'HSP110, apparaît donc nécessaire pour inhiber le développement tumoral et sensibiliser les cellules à la chimiothérapie.

Une meilleure compréhension des fonctions d'HSP110 dans les tissus sains est également nécessaire afin de pouvoir évaluer les effets secondaires potentiels liés à son inhibition, en raison de sa surexpression dans différents tissus. Celle-ci pourrait également, tout comme d'autres HSP, être impliquée dans la différenciation des cellules immunitaires. HSP110 pourrait également jouer des rôles dans des processus non tumoraux tels que les infections et les maladies liées à l'inflammation.

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Annexes

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2009 - 2010 : **MSc,** Cell and Metabolic Biochemistry, pharmacology option, with Honors. Burgundy University, Dijon, France

2006 - 2009 : **Bachelor's Degree**, Biology, Biochemistry option, with honors, Dijon, France

Research experience

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Study of HSP110 fonctions in colorectal cancer :

- Involvement of HSP110 in colorectal cancer cell proliferation through STAT3 pathway
- Role of HSP110 secretion on macrophage skewing in colorectal cancer

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Role of HSP27 and HSP90 in the regulation of the HIF-2α transcription factor in renal cell carcinoma

Jan - june 2011 : **MRes** training period - **UMR INSERM U866 – « Lipides, Nutrition, Cancer »**, Dijon, France

Role of HSP27 and HSP90 in the regulation of the HIF-2α transcription factor in renal cell carcinoma

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Tuning of human ACOX1a and ACOX1b coexpression conditions in E. coli

Technical skills

In vitro Cell culture, Immune cells isolation using magnetic labeling, transfections (plasmids, siRNA), Western blot, quantitative PCR, ELISA (classical or Milliplex technology), Cell proliferation (MTT), Co-immunoprecipitation, Immunofluorescence, DuoLink, Immunohistochemistry, Immunohistofluorescence, Flow cytometry (Cell trace labeling, Membrane receptor expression, BrdU…).

In vivo Sub-cutaneous injection of colorectal cancer cells on mice. Dissection and tumor recovery.

Publications

Articles

Kevin Berthenet, Christophe Boudesco, Ada Collura, Sarah Richaud, Arlette Hamman, Magali Svreck, Kristell Wandherwrick, Laurence Duplomb, Alex Duval*, Carmen Garrido* and Gaetan Jego*. Extracellular HSP110 skews macrophages polarization in colorectal cancer. **Submitted to Cancer Research**

Anaïs Lagrange*, A'dem Bokhari*, **Kevin Berthenet**, Magali Svrcek, Laetitia Marisa, Olivier Buhard, Malorie Greene, Sylvie Dumont, Anastasia R. Goloudina, Jieshuang Jia, Guillaume Marcion, Kristell Wanherdrick, Eric Adriaenssens, Sebastien Causse, Mouna Chouchène, Renaud Seigneuric, Thierry Chassat, Denis S. Biard, Aurélie de Thonel, Jean-François Flejou, Gaetan Jego, Carmen Garrido*, Fabrice Lejeune*, Ada Collura*, Alex Duval*. Mutation of *HSP110* Inhibits Tumor Growth Through a NMD Druggable Pathway in Colorectal Cancer. **Submitted**

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Reviews

Garrido C, Collura A, **Berthenet K**, Lagrange A, Duval A. [Mutation of HSP110 in colorectal cancer: the chaperone paradox]. Med Sci (Paris). 2012 Jan;28(1):9-10.

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Seigneuric R, Mjahed H, Gobbo J, Joly AL, **Berthenet K**, Shirley S, Garrido C. Heat shock proteins as danger signals for cancer detection. Front Oncol. 2011 Nov 10;1:37.

Scientific communications

Oral presentation :

4th European Congress of Immunology (ECI) Annual Congress. 6 – 9 Sept 2015. Vienna, Austria. Extracellular HSP110 from colorectal cancer cells skew macrophages polarization.

Posters :

Forum des Jeunes Chercheurs (FJC). 2013. Dijon, France. HSP110 favors colorectal cancer cells proliferation through STAT3 pathway.

6th International Symposium on Heat Shock Proteins in Biology and Medicine. 3 - 7 Nov 2012. Alexandria, VA, USA. Dual regulation of SPI1/PU.1 transcription factor by heat shock factor 1 (HSF1) during macrophage differentiation of monocytes.

Price and distinction :

Best poster price at the 6th International Symposium on Heat Shock Proteins in Biology and Medicine. 2012.
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Other skills

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ORIGINAL ARTICLE

Dual regulation of SPI1/PU.1 transcription factor by heat shock factor 1 (HSF1) during macrophage differentiation of monocytes

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In addition to their cytoprotective role in stressful conditions, heat shock proteins (HSPs) are involved in specific differentiation pathways, for example, we have identified a role for HSP90 in macrophage differentiation of human peripheral blood monocytes that are exposed to macrophage colony-stimulating factor (M-CSF). Here, we show that deletion of the main transcription factor involved in heat shock gene regulation, heat shock factor 1 (HSF1), affects M-CSF-driven differentiation of mouse bone marrow cells. HSF1 transiently accumulates in the nucleus of human monocytes undergoing macrophage differentiation, including M-CSF-treated peripheral blood monocytes and phorbol ester-treated THP1 cells. We demonstrate that HSF1 has a dual effect on SPI1/PU.1, a transcription factor essential for macrophage differentiation and whose deregulation can lead to the development of leukemias and lymphomas. Firstly, HSF1 regulates *SPI1/PU.1* gene expression through its binding to a heat shock element within the intron 2 of this gene. Furthermore, downregulation or inhibition of HSF1 impaired both *SPI1/PU.1*-targeted gene transcription and macrophage differentiation. Secondly, HSF1 induces the expression of HSP70 that interacts with SPI1/PU.1 to protect the transcription factor from proteasomal degradation. Taken together, HSF1 appears as a fine-tuning regulator of SPI1/PU.1 expression at the transcriptional and post-translational levels during macrophage differentiation of monocytes.

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INTRODUCTION

Exposure to a wide variety of physical and chemical stresses activates the expression of stress response genes coding for heat shock proteins (HSPs). HSPs are molecular chaperones that help the cell to cope with these stressful conditions by mediating correct refolding of the denatured proteins. Besides their well-described role in cell protection under stressful conditions, HSPs have essential roles in a variety of cellular processes, such as cell cycle and apoptosis.[1] HSPs have also demonstrated essential functions in cell differentiation, for example, in erythroblast[2,3] and macrophage differentiation,[4] and their level of expression is tightly regulated during development.[5]

Expression of heat shock genes is regulated by heat shock transcription factors (HSFs), which bind to heat shock elements (HSE) in their promoter region and stimulate their transcription.[5,6] Four members have been identified in mammals: HSF1, HSF2, HSF3 and HSF4. HSF1 is the major stress-responsive family member. In response to stressful stimuli, HSF1 is activated by trimerization and hyperphosphorylation.[5] As a result, HSF1 binds to HSE and activates the transcription of heat shock genes, which results in the accumulation of HSPs such as HSP70 with, as a final outcome, cell protection. HSF1 not only regulates expression of heat shock genes in response to stress but it is also involved in development, by regulating non-heat shock genes.[7]

The transcription factor SPI1/PU.1 is a member of the Ets family proteins expressed in myeloid and B lymphoid cells.[8,9] It has an essential role in the acquisition of the macrophage phenotype by regulating the expression of many myeloid genes, such as those encoding the receptor of the macrophage colony-stimulating factor (M-CSFR, also known as CSF1R), the hemoglobin scavenger receptor CD163, the alpha-M integrin molecule CD11b and the cytokine interleukin-1β (IL-Iβ). The level of SPI1/PU.1 is critical in specifying cell fate, as its deregulation can lead to the development of leukemias and lymphomas.[9–11] Previous reports demonstrated that the proximal promoter of human *SPI1/PU.1* gene and distal regulatory elements located at −15, −14 and −12 kb upstream of the transcription start site, were essential for *SPI1/PU.1* gene regulation.[12,13] In the present study, we identify a dual function of HSF1 in monocyte to macrophage differentiation: HSF1 regulates *SPI1/PU.1* gene expression, and also concomitantly the expression of *Hsp70* gene; the resulting newly synthesized HSP70 protein interacts with SPI1/PU.1 protein to prevent its proteasomal degradation.

MATERIALS AND METHODS

Mice and monocyte purification

The Hsf1-knockout mouse line was derived from animals created by homologous recombination with a gene-targeting vector in embryonic

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stem cells, described by McMillan *et al.*,[14] Hsf1-knockout mice were bred and maintained in a mixed genetic background C57BL/6 J × BALB/c, allowing the production of 15% viable *Hsf1*$^{-/-}$ by crossing *Hsf1*$^{+/-}$ mice. So far, only one study on the lymphoid lineage in spleen, thymus and bone marrow from *hsf1*$^{-/-}$ mice has been performed with no phenotypic outcome.[15] Experiments were performed with the approval of the Ethics committee of the University of Burgundy. Bone marrow cells were extracted from tibias and femurs of wild-type and *Hsf1*$^{-/-}$ mice, labeled with fluorescein isothiocyanate -labeled anti-CD49b, -CD45R, -CD3ε and -ter119 antibodies (Miltenyi Biotec, Paris, France), washed and incubated with anti-fluorescein isothiocyanate microbeads (Miltenyi Biotec). Myeloid cells were then purified with the Automacs and treated with murine M-CSF (10 ng/ml, R&D Systems, Abington, UK). The percentage of CD11b$^+$/CD3$^-$/B220$^-$ myeloid cells, CD19$^+$ cells, Ter119 cells and Lin$^-$Sca1$^+$c-kit$^+$ in the bone marrow, and CD3$^+$ cells and B220$^+$ cells in the blood were determined by flow cytometry. The percentage of macrophages after 4 days of culture was determined by Gr1$^-$ F4/80$^+$ staining. Untouched human monocytes were purified from buffy coats using the monocyte isolation kit II (Miltenyi Biotec).

Cell culture and differentiation

Human monocytes were isolated from healthy and chronic myelomonocytic leukemia (CMML) patients blood samples (IIGR, Villejuif, CHU, Dijon, France), after obtaining a written consent. HeLa, HEK293 T and THP1 cells (DSMZ, Braunschweig, Germany), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. THP1 cells were induced to differentiate into macrophage by exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA, 20 nM, Sigma-Aldrich, St Quentin Fallavier, France). Cell differentiation was assessed by following morphological changes and the expression of cell surface marker CD11b by flow cytometry analysis as described.[16] Primary monocytes were differentiated into macrophages by treatment with M-CSF (100 ng/ml, R&D Systems) and differentiation at different time points was assessed as described.[16] Clonogenic assays were performed collagen-based medium (StemCell Technologies, Grenoble, France) with 100 ng/ml M-CSF. Inhibition of HSF1 was done by quercetin (3 μM) or triptolide (10 nM). Inhibition of HSP70 was done by VER-115008 (100 μg/ml (R&D Systems)). Proteasome inhibition was done by 4 h incubation with MG132 (30 μM (Sigma-Aldrich)) or clasto-lactacystin β-lactone (Sigma-Aldrich). Lipopolysaccharide, 10 ng/ml) was either added or not during the last 6 h of culture. IL-6 and interferon γ-induced protein (IP)-10 were quantified by ELISA (R&D Systems).

Cell transfection and plasmids used

HeLa cells were transfected using JetPEI transfection reagent (Ozyme, St. Quentin en Yvelines, France) and analyzed 48 h after transient transfection. HSP70 full length and HSP70 mutants lacking ATP-binding domain or peptide-binding domain plasmids were obtained after cloning in a hemagglutinin (HA)-tagged vector. A minimal promoter pE1b[17] was inserted upstream of the luciferase gene in pGL3 reporter vector (Promega, Charbonnieres, France) using *SmaI* and *NcoI* enzymes. Then, three constructs have been generated by insertion of SPI1/PU1 intron 2 region upstream of the minimal promoter (synthesized by Genescript, NJ, USA). This region contains the HSE consensus site, either wild type: 5′-CAC AGGGGGTTTTCCAGGAAAGTCTCAGGTCCCAGGAACCTCACGG-3′ or mutated: mutant 1 (M1) 5′-CACAGGATGTTTTTCATTAAAGTTTCAGGTCTCAACTACCAAA CTGG-3′ and mutant 2 (M2) 5′-CACAGGATGTTTTCCAGTAAAGTTTCAGGTCTC AACTACCAAACTGG-3′ flanking by *Kpn*I (5′) and *Nhe*I (3′) enzyme sites.

Immunoblot analysis and immunoprecipitation

Cells were lysed in lysis buffer (1% sodium dodecyl sulfate, 0.4 mM sodium orthovanadate, 10 mM Tris pH 7.4) in the presence of protease inhibitors (Merck, Millipore, Molsheim, France). Proteins were separated and transferred before the analysis with chemiluminescence detection kit (Santa Cruz Biotechnology, Dallas, TX, USA). Nuclear extracts were obtained as previously described.[3] The antibodies used were the anti-HSP70 (StressGen, Enzo Life, Villeurbanne, France), anti-HSC70, anti-Lamin B, anti-MafB, anti-HSF1 and anti-PARP (from Santa Cruz), rabbit polyclonal anti-SPI1/PU.1, anti-NFkB p65, anti-Myc, anti-TLR4 and anti-nucleophosmin-1 from Cell Signaling (Ozyme), Rabbit anti c-Maf from Abcam (Paris, France), and anti-HA from Covance ImmunoTechnologies (Berkeley, CA, USA). Immunoprecipitation was done as previously described.[4]

Immunofluorescence staining

Staining was done as previously described.[4] After fixation and permeabilization, cells were incubated overnight at 4 °C with anti-HSP70 (Tebu-Bio, Le Perray-en-Yvelines, France), anti-SPI1/PU.1 (Ozyme), anti-HSF1 (Tebu-Bio) before incubation with secondary antibodies (Molecular Probe, Leiden, the Netherlands). The nucleus was labeled by 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Images were acquired using the Cell Observer station (Zeiss, Germany).

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed using Millipore EZ-ChIP ChIPKit according to the manufacturer's instructions. Cells (50×10^6) were first fixed by 37% formaldehyde. Glycine was added and cells were lysed in SDS lysis buffer after two washes with PBS. Samples were sonicated (10 pulses of 30 s, Bioruptor's Diagenode, Diagenode, Belgium) and immunoprecipitation of crosslinked protein/DNA was performed with an anti-HSF1 (Ozyme), an anti-dimethyl histone (positive control, Ozyme) or a normal mouse IgG antibody. The complexes were washed five times. After reverse crosslinking, the DNA was purified. Primers for the PCR are available in Supplementary methods. For the experiment shown in Figure 1f, ChIP experiments were performed using ChIP-IT (ActiveMotif, La Hulpe, Belgium). Chromatin, immunoprecipitated by HSF1 (ThermoFisher Scientific, Fremont, CA, USA), phospho Ser5pol II (Active Motif), histone H3K27Ac (Abcam) or SPI1/PU.1 antibodies (Ozyme) was eluted from the magnetic beads after several washes and the crosslinks of these sequentially immunoprecipitated protein–DNA complexes were then reversed. DNA was analyzed by real-time-PCR (Applied Biosystem, Carlsbad, CA, USA) for SPI/PU.1 promoter and intron 2. Primers for the PCR are available in Supplementary Methods.

Electrophoresis mobility shift assay

Cell pellets (25×10^6) were submitted to two rapid freeze–thaw cycles and then lysed (10 mM Hepes pH 7.9, 0.4 M NaCl, 0.1 mM ethylene glycol tetra-acetic acid and 5% glycerol). Proteins were incubated with a (^{32}P)-labeled HSE oligonucleotide (5′-CTAGAACGTTCTAGAAGCTTCGAGA-3′), (^{32}P)-labeled promoter or intron 2 (promoter: 5′-GACTCCAGAAAGTGGAGGCCCCAAG AGG-3′; intron 2: 5′-CACAGGGGGTTTTCCAGGAAAGTCTCAG-3′) and HSF–HSE complexes were separated on a native 4% poly acrylamide gel as described.[18] The components of the retarded complexes were analyzed by supershifting using antibodies against HSF1 (ThermoFisher Scientific).

Luciferase reporter assay

24 h after transfection, luciferase activity was measured using Promega's Dual-Glo luciferase assay System (Promega). The activity of the co-transfected renilla was assayed for normalization. Data were expressed as relative luciferase values (luciferase/renilla ratio).

siRNAs and cell transfection

Monocytes were nucleoporated with small interfering RNA (siRNA) using Amaxa nucleofector kit (Amaxa Biosystems, Gaithersburg, MD, USA). SiRNAs were from Sigma (HSP70: SASI_Hs01_00051449, HSF1: SASI_Hs02_00396607, NFkB p65: SASI_Hs01_00171091, Maf-B: SASI_Hs01_00197228, c-Maf: SASI_Hs01_00202727), from Dharmacon (ThermoFisher Scientific) (HSF1: ON-TARGETplus SMARTpool L-012109-00-0050 and control siRNA ON-TARGETplus NON-targeting Pool D-001810-10-20). After overnight culturing, M-CSF (100 ng/ml) was added to induce macrophage differentiation.

Quantitative real-time-PCR

Primers for SPI1/PU.1 were from ThermoFisher Scientific (AX010537-00-0100), for EGR2 from Tebu-Bio (PPH01478F-200). TLR4 primer sequence is available upon request. GAPDH was used as invariant control.

Yeast double-hybrid assay

Prepared as previously described;[3] details provided in Supplementary Methods.

RESULTS

HSF1 is involved in macrophage differentiation of monocytes

Based on our previous demonstration that HSP90 was involved in M-CSF-induced differentiation of monocytes into macrophages,[4] we explored the consequences of the deletion of *hsf1* gene, which

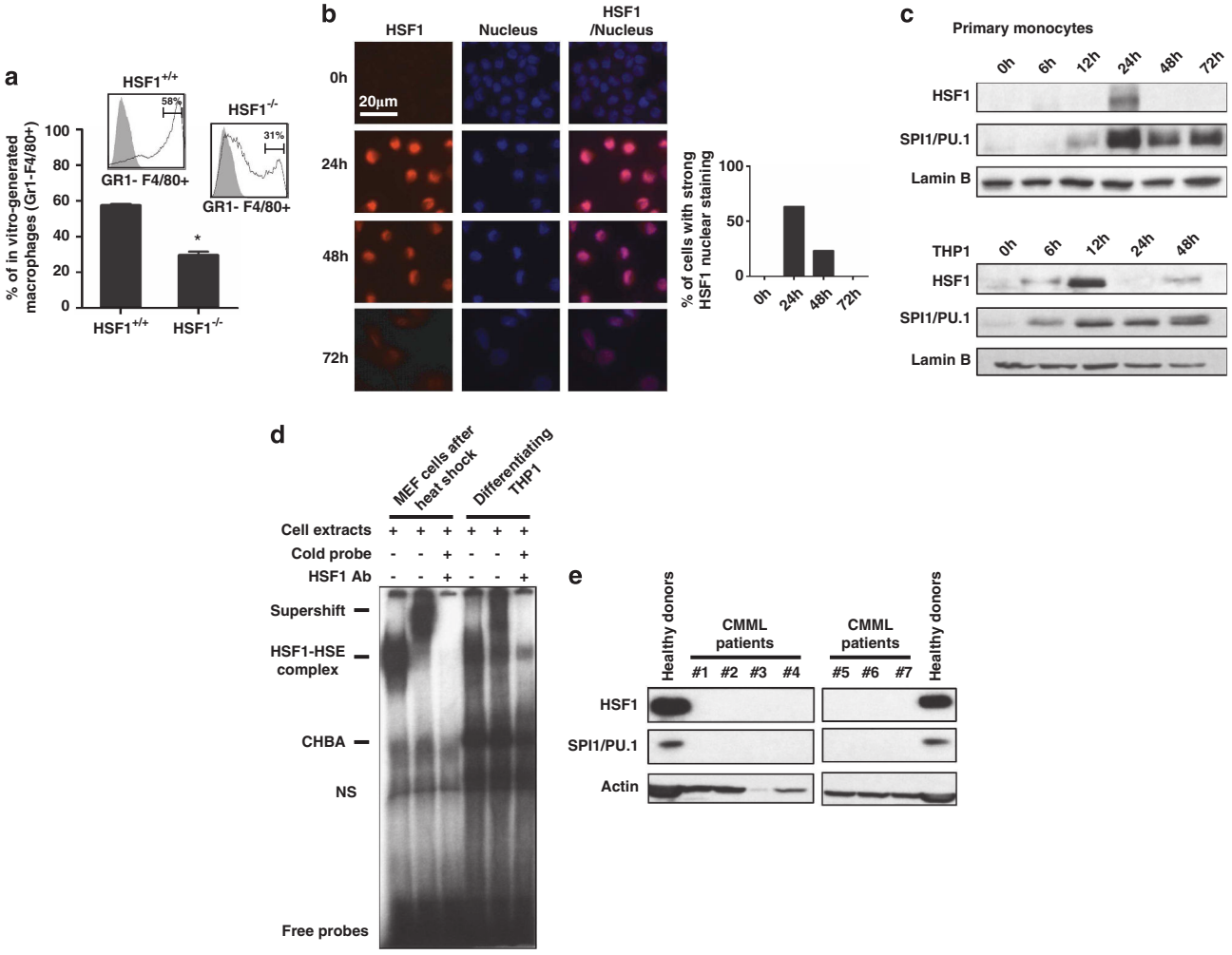

Figure 1. HSF1 is involved in macrophage differentiation of monocytes. (**a**) The percentage of sorted monocytes undergoing *ex vivo* differentiation into macrophages after 4 days of exposure to 10 ng/ml M-CSF was measured in wild-type and *hsf1*$^{-/-}$ mice ($N = 3$/group), *$P < 0.05$. (**b**) Fluorescence microscopy analysis of HSF1 in human peripheral blood monocytes treated with 100 ng/ml of M-CSF for indicated times. Nuclei, labeled with 4′,6-diamidino-2-phenylindole, are stained in blue. Magnification $\times 63$. Right panel, cells labeled that are positive for nuclear HSF1 were counted from 300 cells chosen randomly in different microscopic fields. One representative experiment out of four is shown. (**c**) Immunoblot analysis of HSF1 and SPI1/PU.1 protein expression in nuclear extracts of human monocytes treated with 100 ng/ml of M-CSF (upper), and in THP1 cells treated with 20 nM TPA (lower) for indicated times. Lamin B: loading control. (**d**) Electrophoresis mobility shift assay using the canonical/consensus HSE sequence and supershifting of the HSF–HSE complex with an anti-HSF1 antibody, in the presence of cellular extracts purified from THP1 cells undergoing macrophage differentiation (16 h) or MEF cells exposed to heat shock at 42 °C during 45 min. (**e**) Immunoblot analysis of HSF1 and SPI1/PU.1 protein expression in differentiating monocytes from healthy donors or CMML patients.

encodes the main transcription factor that controls heat shock genes, on monocyte production and differentiation. First, we observed that the percentage of CD11b^{pos} CD3^{neg}B220^{neg} myeloid cells was significantly lower in the bone marrow of *Hsf1*$^{-/-}$ compared with wild-type mice (45% of decrease, $P = 0.02$; $n = 3$) (Supplementary Figure 1A) and formed less M-CSF-dependent colony-forming units (Supplementary Figure 1B, left panel), despite no alteration of the hematopoietic stem cell compartments or other lineages (Supplementary Figures 1C and D). Second, we observed that the master myeloid transcription factor SPI1/PU.1 expression was strongly decreased in total bone marrow cells isolated from *hsf1* knockout compared with wild-type mice (Supplementary Figure 1B, right panel). Third, we noticed that bone marrow monocytes isolated from *Hsf1*$^{-/-}$ mice had a lower ability to differentiate *in vitro* into macrophages (48% of decrease, $P = 0.04$) (Figure 1a). When human peripheral blood monocytes were cultured *ex vivo* in the presence of M-CSF, we observed the accumulation of HSF1 in the cell nucleus after 24 h, and then a time-dependent decrease of the HSF1 staining (Figure 1b), which was confirmed by immunoblot analysis of nuclear extracts (Figure 1c upper panel). Electrophoresis mobility shift assay experiments demonstrated that HSF1 was in a DNA-binding active form as it could bind to a canonical HSE sequence (Figure 1d). We then compared HSF1 expression and localization in human monocytes cultured with M-CSF, GM-CSF or IL-4, three cytokines known to promote macrophage differentiation. Interestingly, GM-CSF and IL-4, similar to M-CSF, strongly induced HSF1 expression within the nucleus at 24 h, followed by a decrease at 48 h, indicating similar kinetics of induction of HSF1 during cytokine-driven macrophage differentiation (Supplementary Figure 2A). These data suggest that HSF1 induction may be a hallmark of macrophage differentiation rather than a phenomenon mediated by a specific cytokine. The transient accumulation of HSF1 in the nucleus was also found in THP1 cells induced to differentiate into macrophage upon phorbol ester exposure (Figure 1c and Supplementary Figures 2B and C). Interestingly,

the expression of HSF1 followed the same kinetics as SPI1/PU.1 (Figures 1b and c and Supplementary Figures 2B and C). To give some insight into the possible clinical relevance of HSF1 in monocytes differentiation, we studied HSF1 expression in CMML patients. This disease is frequently associated with defective monocyte differentiation.[19] The difficulty of patients' monocytes to differentiate upon M-CSF exposure, associates with no or very little expression of both HSF1 and SPI1/PU.1 (Figure 1e and Supplementary Figure 2D). The concomitant decrease in both SPI1/PU.1 and HSF1 in this clinical setting enforces the hypothesis that the two factors may have a coordinated role in monocyte differentiation and prompted us to explore whether HSF1 could control PU.1 expression.

SPI1/PU.1 gene contains several HSE-like sequences including in its promoter and, particularly well conserved, in its second intron, as determined by Genomatix software (http://www.genomatix.de). We observed by electrophoresis mobility shift assay experiment that HSF1 is able to bind to the HSE consensus site found in intron 2 but not in the promoter of *SPI1/PU1* gene in the differentiating monocytic cells (Supplementary Figure 3A). ChIP, using an anti-HSF1 antibody and primers surrounding the putative HSE sequences, demonstrated that HSF1 could bind to the *SPI1/PU.1* gene within the intron 2, but not within the promoter during primary monocyte differentiation (Figure 2a). A ChIP–qPCR was performed to measure the enrichment of HSF1 binding at the promoter and intron 2 regions of *SPI1/PU1* gene after 24 h of differentiation (Figure 2b) and Supplementary Figures 4 and 5). In parallel, we monitored two markers of active chromatin conformation surrounding the putative HSEs—the phosphorylated RNA polymerase II on serine 5 (that is, the active polymerase) and the acetylated histone H3 on lysine 27 (H3K27ac).[20] We observed that HSF1 binding to *SPI1/PU-1* intron 2 region correlated with the recruitment of active RNA polymerase II and the presence of H3K27ac. In contrast, enrichment of HSF1 was not detected at the promoter region, which also contained a HSE motif, although active RNA polymerase II was present (Figure 2b). Furthermore, the -15, -14 and -12 kb upstream regulatory elements of the human *SPI1/PU.1* gene[12] were tested with no significant HF1 binding observed (Figure 2b). These results were confirmed in THP1 cells treated for 16 h with phorbol esters (Supplementary Figures 3B and C). In order to functionally test whether *SPI1/PU.1* intron 2 had transcriptional enhancing capacity, we used a luciferase reporter gene driven by the minimal promoter pE1b[17] and inserted the intron 2 sequence of *SPI1/PU-1* containing the putative HSE (wild-type int2) or two mutated versions (M1 and M2, Figure 2c) upstream of this minimal promoter. HEK293 cells were co-transfected with a construct encoding wild-type HSF1 (HSF1 Myc)—or with the mock vector—and a luciferase reporter containing the wild-type intron 2 sequence (Figure 2c). In these

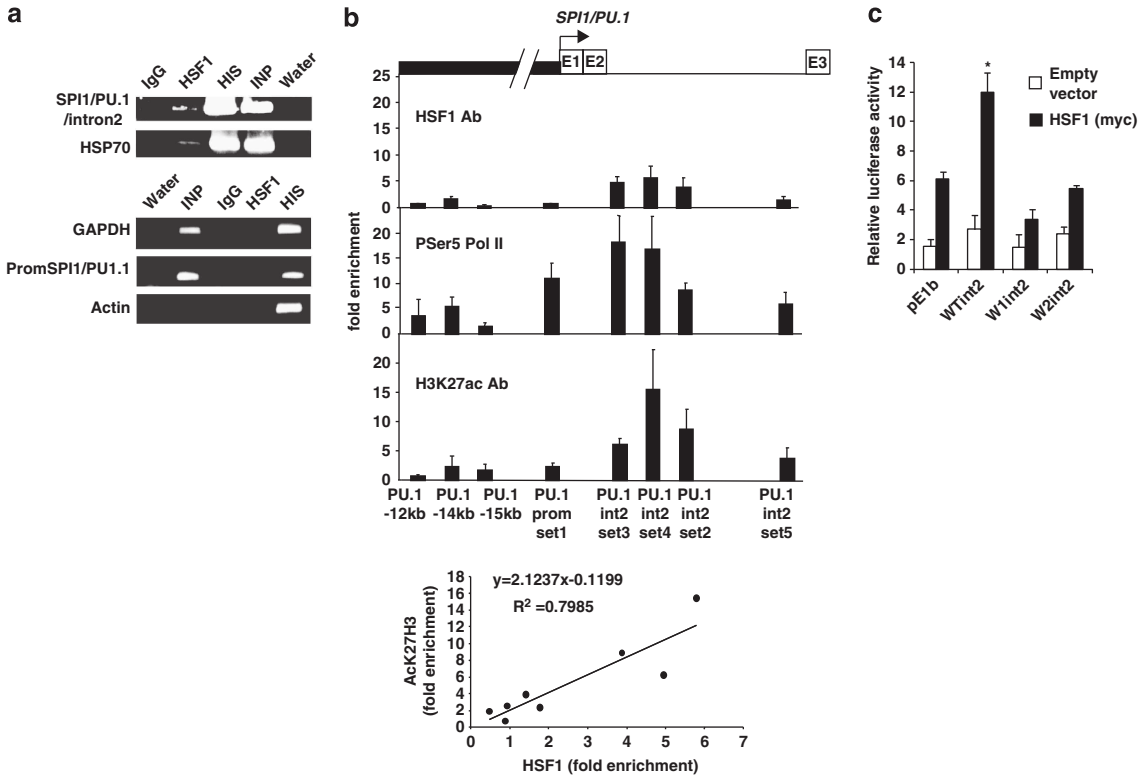

Figure 2. HSF1 binds to SPI1/PU.1 intron 2. (**a**) Representative ChIP experiments from primary human monocytes treated for 24 h with M-CSF as above. PCR was used to detect DNA fragments encompassing the HSE located in *SPI1/PU.1* gene intron 2, *SPI1/PU.1* gene promoter, *Hsp70* promoter (positive control) and *GAPDH* promoter (negative control) after ChIPwith an anti-dimethyl histone (HIS), an anti-HSF1 (HSF1), or a non-relevant (IgG) antibody. Input: total cell lysate (INP), water: no lysate. Representatives of at least three independent experiments are shown. (**b**) Quantification of the enrichment of HSF1-binding in ChIP experiments followed by RT–qPCR analysis from primary human monocytes treated for 24 h with M-CSF. Chromatin immunoprecipitated by HSF1, phospho Ser5pol II or histone H3K27Ac antibodies was analyzed by real-time-PCR with five couples of primers (set1–5) corresponding to HSE-containing regions within the *SPI1/PU.1* promoter (set1) and four putative HSE in intron 2 (set2–5), and with three couples of primers corresponding to three known upstream regulatory elements of the *SPI1/PU.1* gene. Calculated enrichment was done as a ratio of the amplification efficiency of the ChIP sample over that of the IgG (upper panels). Correlations between HSF1 fold enrichment and AcH3K27 fold enrichment found in the ChIP–qPCR experiments (lower panel). (**c**) Luciferase reporter experiment in HEK 293T cells. Cells co-transfected with renilla reporter (internal control) and the different luciferase reporters (containing HSP70 promoter or, minimal promoter pE1b, minimal promoter pE1b associated to wild-type *SPI1/PU.1* intron 2 or mutated versions (M1 and M2)) together with a wild-type HSF1 constructs (HSF1 Myc) or the empty vector, were analyzed after 24 h. Shown are relative luciferase values (luciferase/renilla ratio). $^*P < 0.05$.

conditions, we observed a threefold induction in luciferase activity. Notably, this increase was not observed when cells were co-transfected with the luciferase reporter construct containing the intron 2 sequence mutated in the HSE (M1 or M2) or containing the minimum SPI1/PU.1 promoter alone (Supplementary Figure 3D). Overall, these results demonstrate that active HSF1 binds to a HSE sequence within the intron 2 of SPI1/PU.1 gene in monocytes undergoing M-CSF-induced macrophage differentiation.

HSF1 inhibition prevents SPI1/PU.1 expression

Exposure of phorbol esters-treated THP1 cells to HSF1 inhibitors, either 10 μM quercetin or 10 nM triptolide, concentration at which both the molecules were able to block the HSF1-dependent heat shock response (Supplementary Figure 6A), prevented SPI1/PU.1 induction in THP1 cells (Figure 3a). Triptolide also inhibited the induction of SPI1/PU.1 protein levels and of the well-known HSF1 target, HSP70, in human primary monocytes treated with M-CSF for 48 h (Figure 3b). This effect is likely not related to an increase in the proteasomal degradation of the SPI1/PU.1 protein, as MG132-mediated inhibition of the proteasome did not prevent the decrease of expression observed upon triptolide and quercetin exposure (Figures 3a and b, and Supplementary Figures 6B–D). Moreover, HSF1 inhibition by triptolide prevented the increase in *SPI1/PU.1* mRNA measured in human monocytes after 24 h of M-CSF (Figure 3c). The *SPI1/PU.1* mRNA and protein accumulation in M-CSF-treated monocytes was inhibited by downregulation of *HSF1* gene expression (31% decrease in mRNA expression, $P = 0.007$, $n = 4$) (Figures 3d and e). *TLR4* and *EGR2* mRNA levels, whose expression is controlled by SPI1/PU.1, were also decreased

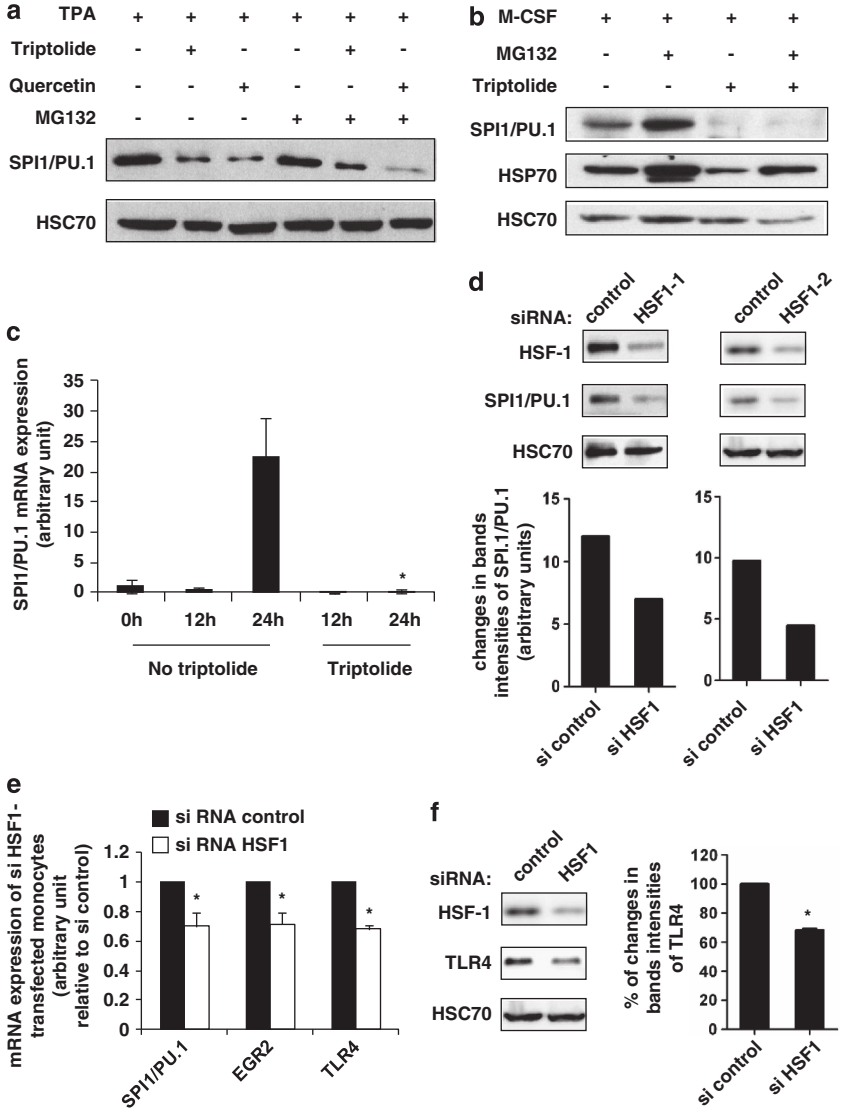

Figure 3. HSF1 regulates SPI1/PU.1 expression. (**a**, **b**) Immunoblot analysis of HSP70 and SPI1/PU.1 in THP1 cells treated for 24 h with 20 nM TPA (**a**) and in human monocytes treated for 48 h with 100 ng/ml of M-CSF (**b**) When indicated, the cells were treated with 10 nM triptolide or 10 μM quercetin or MG132 (30 μM for 4 h before cell lysis). HSC70: loading control. (**c**) qPCR analysis of *SPI1/PU.1* gene expression in human monocytes left untreated (0 h) or treated for indicated times with M-CSF, without or with 10 nM triptolide. (**d**) Immunoblot analysis of HSF1 and SPI1/PU.1 in human monocytes transfected by a universal control siRNA or with two different siRNAs that targets HSF1 (HSF1-1 and HSF1-2), then treated for 48 h with 100 ng/ml of M-CSF. HSC70: loading control. (**e**) qPCR analysis of SPI1/PU.1, EGR2 and TLR4 mRNA in monocytes transfected with a universal control siRNA or a HSF1-specific siRNA and treated with M-CSF for 48 h. Mean values (± s.d.) of at least three independent experiments are shown. *$P < 0.05$. (**f**) Immunoblot analysis of HSF1 and TLR4 in human monocytes transfected by a universal control siRNA or with a siRNA that targets HSF1, and then treated for 48 h with M-CSF (100 ng/ml). HSC70: loading control. Right panel shows the percentage of change in TLR4 band intensity from three independent experiments, *$P < 0.05$.

in the same proportions by HSF1 siRNA (29% decrease, $P=0.025$, $n=3$ and 32% decrease, $P=0.0023$, $n=3$, respectively) (Figure 3e). Accordingly, TLR4 protein level decreased in SPI1/PU.1-depleted monocytes (Figure 3f) and the amount of SPI1/PU.1 bound to TLR4 promoter was strongly decreased in the HSF1-depleted monocytes (Supplementary Figure 6E). Thus, HSF1

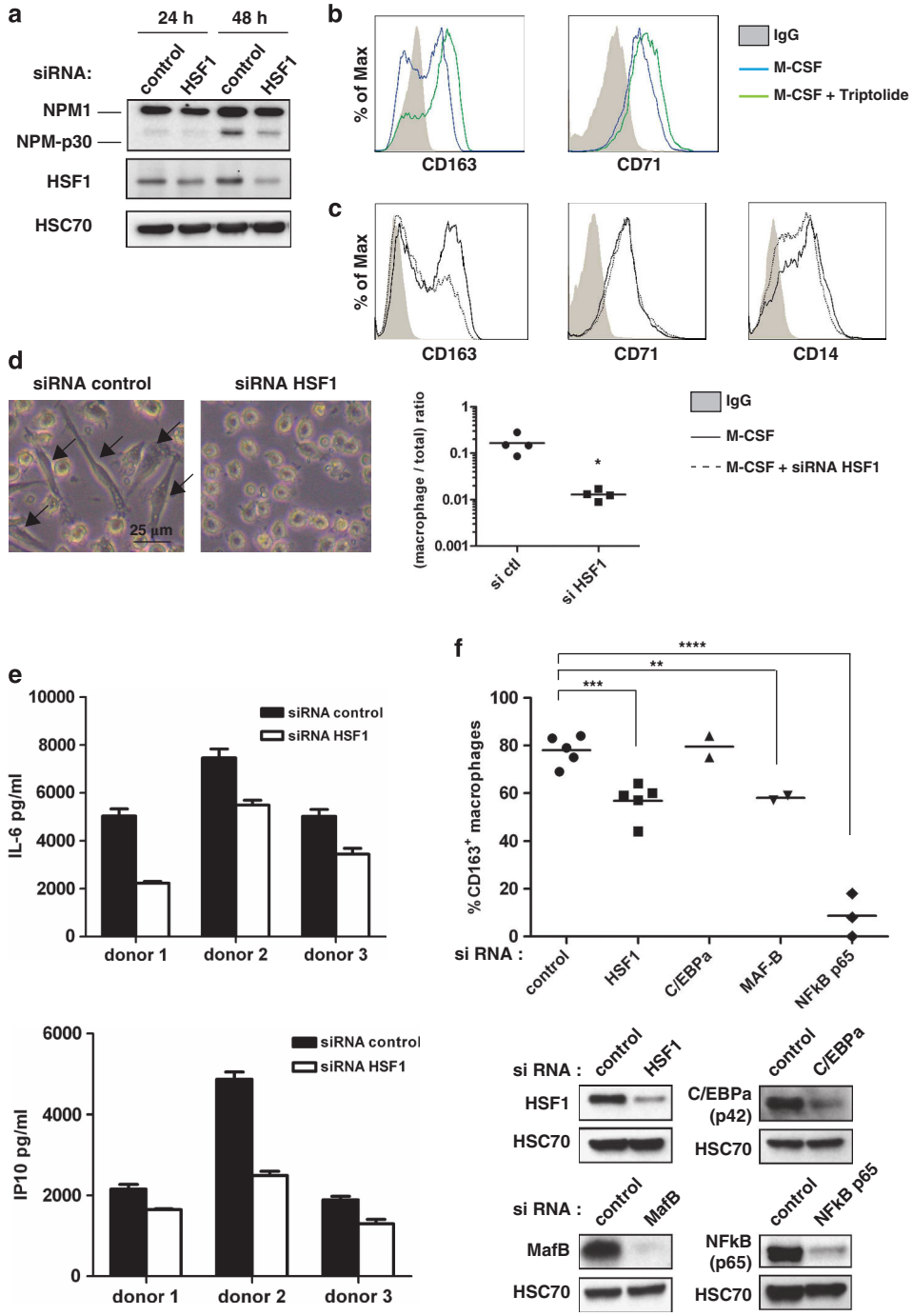

Figure 4. HSF1 inhibition decreases macrophage differentiation. (**a**) Immunoblot analysis of HSF1 and nucleophosmin-1 in human monocytes transfected by a universal control siRNA or with siRNA that targets HSF1, then treated for 24 h and 48 h with 100 ng/ml of M-CSF. HSC70: loading control. (**b**, **c**) Flow cytometry analysis of CD163, CD71 and CD14 expression on monocytes treated for 48 h with M-CSF, without or with 10 nM triptolide (**b**) or transfected with a universal control or a HSF1-targeting siRNA, then treated for 48 h with 100 ng/ml M-CSF (**c**). (**d**) (left) Phase contrast examination of primary monocytes transfected with a universal control siRNA or with a HSF1 siRNA, then treated for 48 h with M-CSF. Arrows show macrophage-like morphology. Magnification $\times 20$. Right panel, the ratio of cells presenting macrophage-like morphology was determined from 300 cells chosen randomly in different microscopic fields ($n=4$) $^*P<0.05$. (**e**) ELISA showing variations in IL-6 and IP-10 concentrations in macrophages differentiated from monocytes isolated from three different donors and transfected with a universal control siRNA or a HSF1-specific siRNA, and treated with lipopolysaccharide. (**f**) Percentage of CD163 positive macrophages differentiated from monocytes transfected with an universal control, HSF1-, C/EBPa-, MafB- or NFkB p65-targeting siRNA, then treated for 48 h with 100 ng/ml M-CSF (upper panel). Immunoblot analysis of HSF1, C/EBPa, MafB and NFkB p65 in above described human transfected monocytes (lower panels).

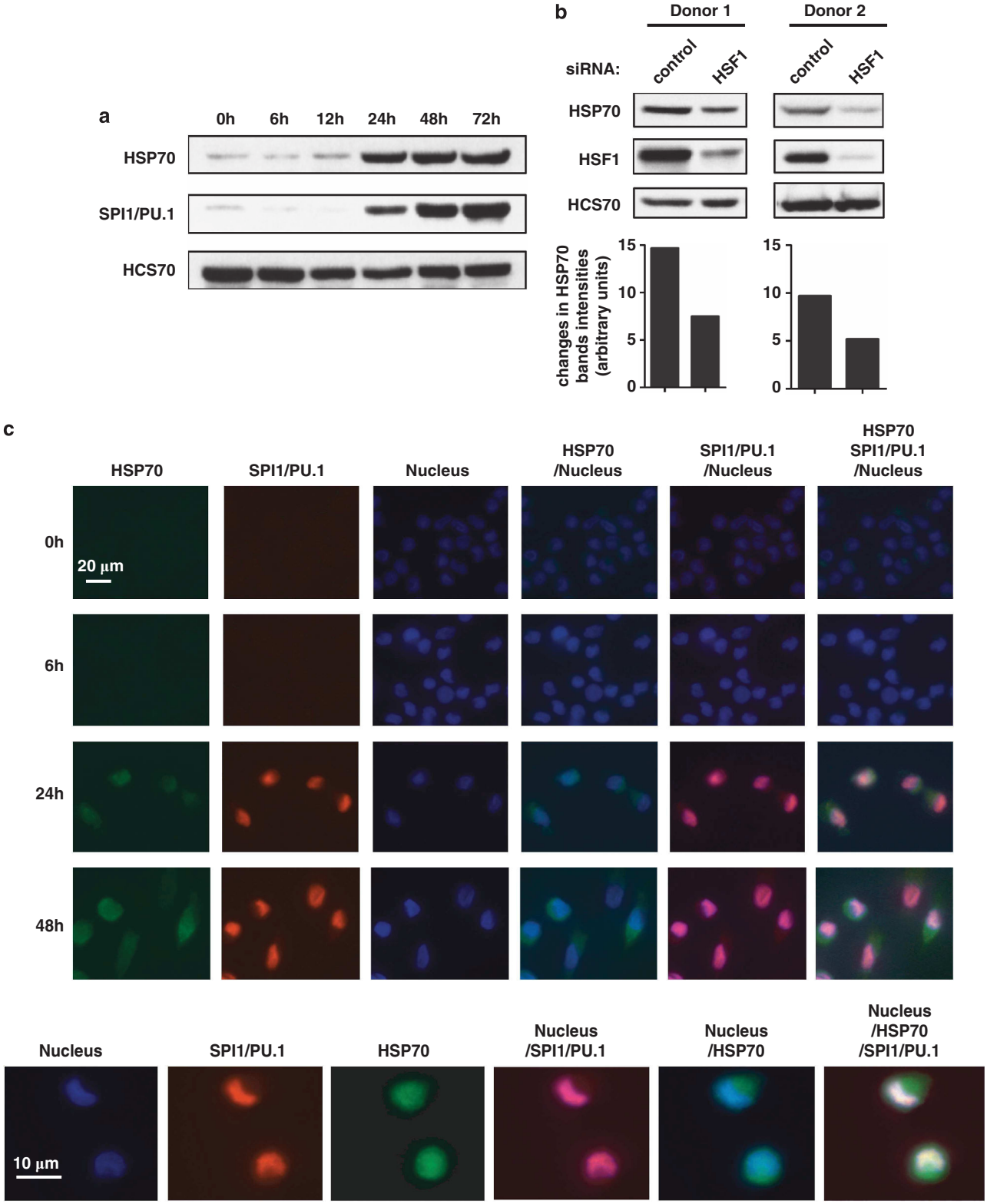

Figure 5. HSP70 induction during macrophagic differentiation. (**a**) Immunoblot analysis of HSP70 and SPI1/PU.1 in monocytes either left untreated or treated with M-CSF for indicated times. HSC70 was used as a loading control. (**b**) Immunoblot analysis of HSF1 and HSP70 in monocytes from two independent donors transfected by a universal control siRNA or with a siRNA that targets HSF1 and treated 48 h with M-CSF. HSC70 was used as a loading control. (**c**) Fluorescence microscopy analysis of HSP70 and SPI1/PU.1 in monocytes either left untreated or treated with M-CSF for indicated times (lower panels), for 24 h (lower panels). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Magnification $\times 63$ (upper panels), magnification $\times 100$ (lower panels). Representatives of at least three independent experiments are shown.

inhibition prevents the increase in the expression of *SPI1/PU.1* gene and protein during macrophage differentiation.

Next, we determined whether HSF1 inhibition/depletion could affect M-CSF-induced differentiation of primary human monocytes. We recently demonstrated that nucleophosmin-1 was cleaved by caspases and cathepsins along with this differentiation process to generate a 30 kDa N-terminal fragment.[21] SiRNA-mediated HSF1 downregulation decreased the cleavage of nucleophosmin-1, observed 48 h after the induction of differentiation, suggesting that the process was impaired (Figure 4a). Furthermore, treatment of cells with triptolide decreased the percentage of cells expressing the specific macrophagic marker CD163 ($-42\% \pm 13\%$, $n=3$, $P<0.005$) without affecting CD71 expression whose increase occurs before the nuclear translocation of HSF1 (Figure 4b). Similarly, HSF1 siRNA induced a decrease in the percentage of cells expressing CD163 ($-32 \pm 10\%$, $n=4$, $P<0.005$), in the mean fluorescence ratio of CD163 expression ($39 \pm 9\%$, $n=4$, $P<0.005$), and in the percentage of CD14 expressing cells (by $15 \pm 4\%$, $n=3$, $P<0.05$, Figure 4c) without affecting cell death (Supplementary Figure 7A). A similar inhibition of TPA-induced differentiation was observed in THP1 cells upon triptolide or quercetin treatment (Supplementary Figure 7B). This inhibitory effect was also demonstrated by the lack of induction of the fibroblast-like shape that characterized this differentiation pathway (Figure 4d and Supplementary Figures 7C and D). Furthermore, HSF1 siRNA induced a decrease in the production of two typical macrophage proinflammatory cytokines, IL-6 and IP-10 (Figure 4e). Altogether, these results indicated that HSF1 depletion or inhibition affected several features of M-CSF-induced differentiation of monocytes.

We next compared the effect of HSF1 knockdown with that of C/EBPa, Maf-B and NFkB p65, three transcription factors involved in myeloid and/or macrophage differentiation. Depletion of Maf-B induced a decrease in cell differentiation that was similar to that obtained upon the depletion of HSF1 ($\sim 40\%$ in both cases, Figure 4f). Upon NFkB p65 depletion, we observed a strong decrease in the cells' differentiation together with a dramatic increase in cell death. Finally, C/EBPa deletion had no impact on cell differentiation (Figure 4f). These results confirm the role of HSF1 in the macrophage differentiation process.

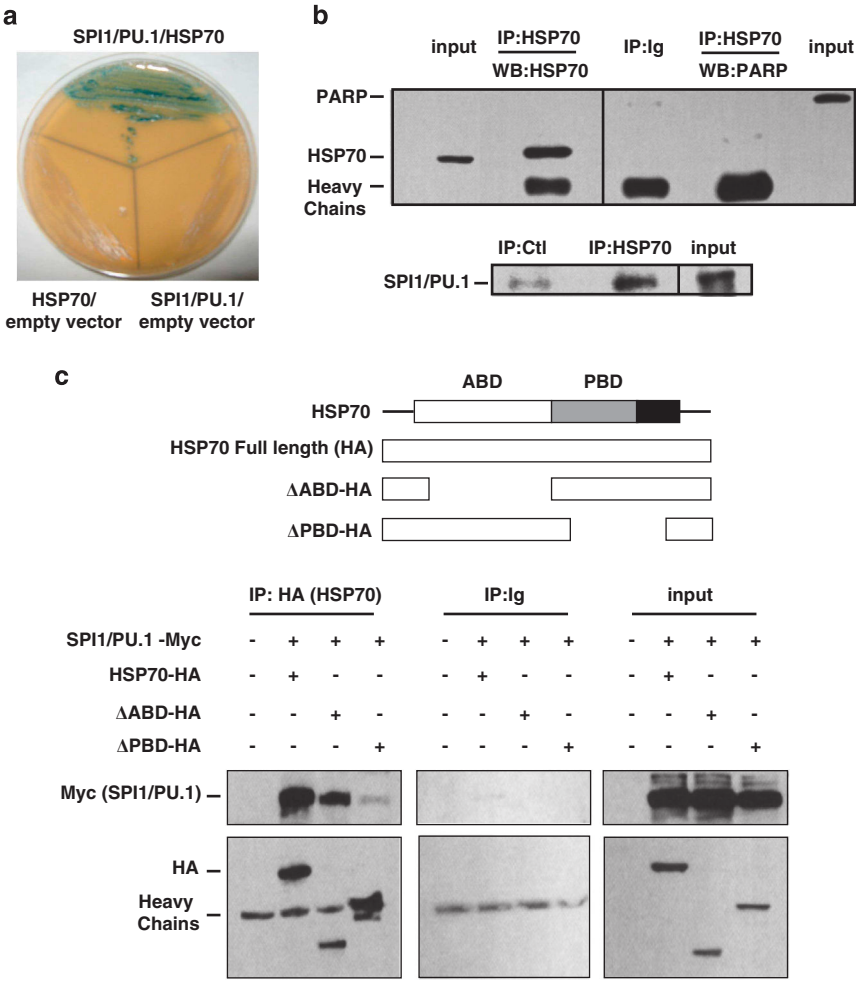

Figure 6. HSP70 interacts with SPI1/PU.1 in differentiating monocytes. (**a**) Yeast double-hybrid assay that detects a direct interaction of HSP70 with SPI1/PU.1 (blue diploid yeast cells). Each section contains diploid yeast cells resulting from an independent yeast mating experience with the corresponding bait protein and a prey protein. The empty vector pGADT7 and pGBKT7 are used as negative controls. (**b**) Coimmunoprecipitation of SPI1/PU.1 and HSP70 in THP1 cells, treated for 24 h with 20 nM TPA, using a HSP70 (IP HSP70) or a non-relevant (IP IgG) antibody. Immunodetection of PARP was used as a control for the specificity of HSP70 binding to SPI1/PU.1. (**c**) HeLa cells were co-transfected with a plasmid coding SPI1/PU.1 fused with a Myc Tag and a plasmid coding HSP70 full-length or deleted for the ATP-binding domain, HSP70ΔADB, or from the peptide-binding domain, HSP70ΔPBD, and fused to a HA tag. Immunodetection of SPI1/PU.1 (Myc) and HSP70 (HA) followed after immunoprecipitation with an anti-HA antibody or with a non-relevant (IP IgG). One representative experiment is shown ($n=3$).

HSP70 prevents the proteasomal degradation of SPI1/PU.1 in differentiating monocytes

Under stressful conditions, HSF1 induces the expression of several HSPs, with HSP70 being the most inducible of these proteins. The expression of HSP70 increases 24 h after the beginning of M-CSF treatment, lasting several days (Figure 5a and Supplementary Figure 8A), and, as expected, this increase is significantly reduced by triptolide treatment (Figure 3b) or siRNA-mediated HSF1 depletion (Figure 5b). Immunofluorescence analysis of these primary monocytes (Figure 5c and Supplementary Figure 8B) and THP1 cells (Supplementary Figure 8C) demonstrated that HSP70, although present everywhere in the cell, accumulated in the nucleus like SPI1/PU.1 did. Using a yeast double-hybrid approach, we show that HSP70 and SPI1/PU.1 do interact directly (Figure 6a). Coimmunoprecipitation experiments demonstrated that endogenous HSP70 associated with SPI1/PU.1 in 24 h-TPA-treated THP1 cells, but not with other nuclear proteins like PARP, showing the specificity of binding (Figure 6b). HeLa cells were co-transfected with a vector encoding Myc-tagged SPI1/PU.1 and a vector encoding HA-tagged HSP70, either full length, or lacking its ATP-binding domain (HSP70ΔABD), or its peptide-binding domain (HSP70ΔPDB). Coimmunoprecipitation experiments with an anti-HA antibody demonstrated that, although SPI1/PU.1 interacted with full-length HSP70 or HSP70ΔADB construct, it failed to interact with HSP70ΔPDB (Figure 6c), indicating that the peptide-binding domain of HSP70 was involved in its interaction with SPI1/PU.1. The levels of both ectopically expressed SPI1/PU.1 in HeLa cells (Figure 7a) and endogenous SPI1/PU.1 in primary monocytes (Figure 7b) were strongly reduced by HSP70 knockdown. SPI1/PU.1 half-life study in the presence of the protein synthesis inhibitor cycloheximide, with or without the HSP70 inhibitor VER-155008, further proved the role of HSP70 stabilizing SPI1/PU.1 (Figure 7c). Furthermore, in the presence of the proteasome inhibitor MG132 (Figure 7b) or lactacystin (Supplementary Figure 6C), SPI/PU.1 levels were restored suggesting that HSP70 stabilized SPI1/PU.1 by interfering with its proteasomal degradation. Confirming this result, ubiquitinated SPI1/PU.1 accumulated in HSP70-depleted cells treated with MG132 (Figure 7d). Together with a decrease in SPI1/PU.1, HSP70 downregulation also induced an inhibition of macrophage differentiation, as assessed by the reduced expression of CD163 ($-31 \pm 9\%$, $n=3$, $P<0.05$) and CD14 (Figure 7e). Altogether, these results suggest that SPI1/PU.1 strictly requires HSP70 for its stabilization during macrophage differentiation.

DISCUSSION

A tight regulation of the differentiation process is required during hematopoiesis, and deregulated differentiation has been identified in many hematologic malignancies. Accumulated evidences suggest that HSPs are instrumental in this regulation.[22] HSPs have been shown to promote differentiation through different mechanisms, including the regulation of the nuclear/cytosolic shuttling of proteins, such as HSP90 that allows c-IAP1 nuclear export during monocyte-macrophage differentiation. HSPs can also prevent degradation of critical proteins involved in the differentiation process. Indeed, HSP90 can also prevent ubiquitination and subsequent proteosomal degradation of

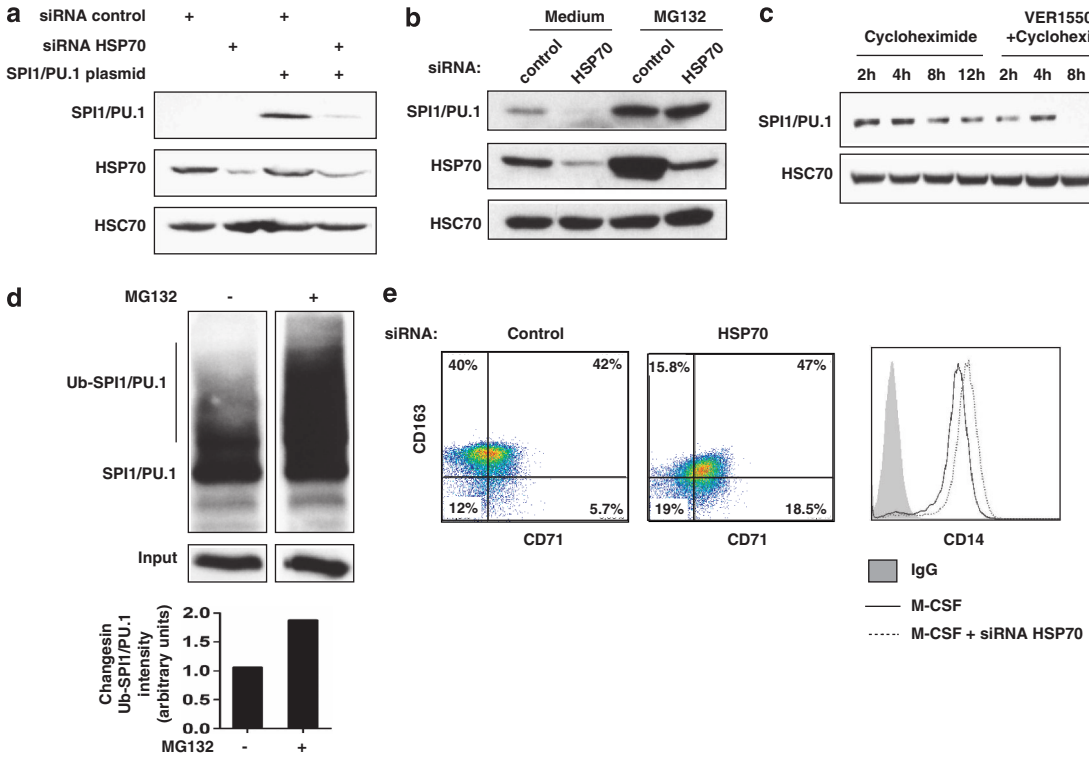

Figure 7. Depletion of HSP70 favors SPI1/PU.1 degradation and impairs primary monocytes differentiation. (**a**) Immunoblot analysis of SPI1/PU.1 and HSP70 in Hela cells co-transfected or not with a plasmid coding SPI1/PU.1, a universal control siRNA or a siRNA that targets HSP70. (**b**) Immunoblot analysis of HSP70 and SPI1/PU.1 in monocytes transfected with a universal control siRNA or a HSP70 siRNA and treated 48 h with M-CSF, with or without MG132 (30 μM) during the last 4 h of the culture. (**c**) Immunoblot analysis of SPI1/PU.1 in THP1 cells treated by cycloheximide (100 μg/ml), in the presence or absence of VER-115008 (10 μg/ml), during the indicated times. (**d**) Immunoprecipitation of ubiquitin was followed by SPI/PU.1 immunobloting in the above used HeLa cells transfected with SPI/PU.1 and a HSP70 siRNA, in the presence or not of MG132 during the last 4 h of the culture. Lower panel shows by densitometry the change in Ub-SPI.1/PU.1 bands intensity. (**e**) Flow cytometry analysis of CD71, CD163 and CD14 expression on monocytes transfected with a universal control siRNA or a siRNA that targets HSP70, and treated 48 h with M-CSF.

c-IAP1, and HSP70 prevents caspase 3-mediated cleavage of GATA-1 during erythropoiesis.[2] Conversely, in myelodysplatic syndromes, defective nuclear localization of HSP70 is associated with dyserythropoiesis.[23] Furthermore, HSF1 controls several genes involved in immune responses, and lipopolysaccharide-stimulated macrophages isolated from HSF1-null mice do not adhere to culture plates, whereas those from wild-type mice do, suggesting a dysfunction of macrophages in HSF1-null mice.[15,24–26] However, the role of HSF1 in the differentiation process of monocytes has not been explored. We show here that HSF1 affects several of the phenotypic changes associated with M-CSF-induced differentiation of monocytes at transcriptional and post-transcriptional levels, through the joint expression of both SPI1/PU.1 and HSP70 (Figure 8).

SPI1/PU.1 has a critical role in myeloid hematopoiesis and its expression must be tightly regulated. We observed that the promoter and the second intron of *SPI1/PU.1* gene bear several HSE-like sequences, but HSF1 binds only to the second intron as demonstrated by ChIP experiments. In support of this finding, two genetic polymorphisms within the intron 2 of *SPI1/PU.1* gene have been recently associated to systemic lupus erythematosus, suggesting that this region is important for the regulation of *SPI1/PU.1* gene expression.[27] Intron-located *cis*-acting elements have previously been described in several genes such as IL-4, Ig or c-fms[28,29] and are involved in chromatin structure and accessibility of transcription factors. In this respect, it is interesting to note that HSF1 can also open the chromatin structure of the IL-6 promoter to facilitate binding of activators or repressors.[26] Further, more than 50% of HSF4-binding sites map to introns or exons in the genome, whereas only 5% map to promoter proximal regions.[30] As inhibition/depletion of HSF1 decreases SPI1/PU.1 mRNA levels, we propose the intron 2-located HSE as a new intron transcriptional regulator.

HSF1 involvement in hematopoietic cell differentiation is probably not limited to macrophages as dendritic cell differentiation is also impaired upon triptolide treatment of GM-CSF/IL-4-cultured monocytes.[31] Although not explored, this effect might also be related to *SPI1/PU.1* gene regulation, as dendritic cell differentiation requires a high level of SPI1/PU.1 expression.[32] Generation of different hematopoietic lineages is controlled by cooperative or competitive interactions with other transcription factors. This could be illustrated by the progranulocyte transcription factor C/EBPα that inhibits *SPI1/PU.1* transactivation.

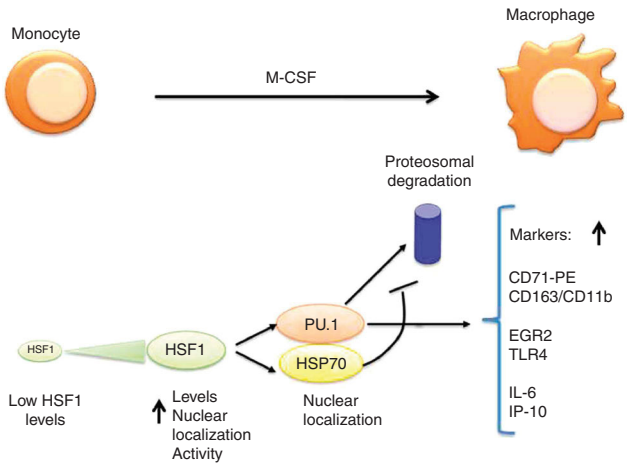

Figure 8. Proposed model for HSF1 regulation of macrophage differentiation. Upon monocytes differentiation, HSF1 is activated and induces the transcription of both genes *SPI1/PU.1* and *HSP70*. At its turn, the chaperone HSP70 interacts with SPI1/PU.1 assuring its stability, thereby allowing macrophage differentiation. Included are some macrophage markers studied in this work.

Given our finding about SPI1/PU.1 control of expression and stability by HSF1, it is not surprising that HSF1 is also a competitive inhibitor of NF-IL-6 binding to the G-CSF promoter, thus favoring myeloid differentiation instead of granulopoiesis.[33]

In addition to the *SPI1/PU.1* promoter, distal regulatory elements located at −15, −14 and −12 kb upstream of the *SPI1/PU.1* gene in the mouse are indispensable to the expression and lineage-restricted auto-regulation of *SPI1/PU.1*. Therefore, it would be of interest to determine the nature of the crosstalk between myeloid-restricted transcription factors that bind these distal regulatory elements and HSF1. HSP70 expression during stressful conditions is controlled by HSF1 binding to HSE. We observed that HSP70 is also induced by HSF1 during macrophage differentiation as silencing of HSF1 by siRNA also decreases HSP70 expression. Furthermore, HSP70 that is barely expressed at basal level in monocytes is strongly upregulated, like HSF1 and SPI1/PU.1, within 24 h of M-CSF treatment. The involvement of HSP70 in the differentiation process has been described in erythropoiesis where it protects GATA-1 from caspase 3-mediated proteolysis through its peptide-binding domain.[2] Similarly, we have found here that upregulated HSP70 translocates to the nucleus and binds to SPI1/PU.1 through its peptide-binding domain, thereby preventing SPI1/PU.1 proteasomal degradation. Given that the expression and state of activation of HSF1 is transient (Figure 1c), the stabilization of SPI1/PU.1 through its interaction with HSP70 is, therefore, necessary to sustain the action of SPI1/PU.1 on its multiple target genes.

Cell differentiation, which as a developmental process can be envisioned also as a stress, might need subtle 'sensors' to initiate rewiring of transcription factor networks. This role could be performed by transcription factors like HSF1, which could trigger the upregulation of other transcription factors like SPI1/PU.1. In support of this idea, transcription factors of the E2F family have been identified as a HSF1 target in mouse oocytes.[34] In addition, it was recently demonstrated that the role of HSF1 in cancer relies on a transcriptional program that is distinct from heat shock response. In this program, HSF1 also regulates the expression of a number of transcription factors like NFATc, STAT1 or STAT3.[35] The crosstalk between the sensor abilities of HSF1 and its ability to remodel transcription pathways therefore represents an elegant and pivotal way to trigger cellular differentiation.

HSF1 effect in SPI1/PU.1 regulation during macrophages' differentiation may have an impact in a clinical setting, given the preliminary results shown in this work in CMML, the most frequent myelodysplastic/myeloproliferative disorder. The difficulty of patients' monocytes to differentiate upon M-CSF exposure associates with no or very little HSF1 and SPI1/PU.1 expression. The usefulness of testing this important decrease in HSF1 and SPI1/PU.1 as a diagnosis or prognostic marker will deserve a specific prospective study, that is, will be tested in the setting of a European prospective clinical trial to be initiated in the first months of 2014. From a therapeutic point of view, another important question that we will explore in depth is whether the re-expression of HSF1 is sufficient to activate SPI1/PU.1 expression and/or to rescue myeloid differentiation in CMML cells. This future study will strengthen the knowledge about the transcriptional link between HSF1 and SPI1/PU.1.

In conclusion, we have identified a new mechanism of SPI1/PU.1 regulation by HSF1 and HSP70 that act synergistically during macrophage differentiation of monocytes (Figure 8). This finding highlights a transcriptional activity of HSF1 that goes beyond the regulation of HSPs and cytokines and suggests a critical role in hematopoiesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

GJ, DL, ADT, ES, CG, designed the study and experiments; GJ, DL, ADT, ND, AH, KB, P-SB, GW, AJ, LD and ALM performed the experiments; VLM, EC, CP, PB and ND provided samples and expertise; GJ, DL, ADT, ND, AH, PB, AH and LD analyzed the experiments; CG directed the work.

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Oncogenic extracellular HSP70 disrupts the gap-junctional coupling between capillary cells

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ABSTRACT

High levels of circulating heat shock protein 70 (HSP70) are detected in many cancers. In order to explore the effects of extracellular HSP70 on human microvascular endothelial cells (HMEC), we initially used gap-FRAP technique. Extracellular human HSP70 (rhHSP70), but not rhHSP27, blocks the gap-junction intercellular communication (GJIC) between HMEC, disrupts the structural integrity of HMEC junction plaques, and decreases connexin43 (Cx43) expression, which correlates with the phosphorylation of Cx43 serine residues. Further exploration of these effects identified a rapid transactivation of the Epidermal Growth Factor Receptor in a Toll-Like Receptor 4-dependent manner, preceding its internalization. In turn, cytosolic Ca^{2+} oscillations are generated. Both GJIC blockade and Ca^{2+} mobilization partially depend on ATP release through Cx43 and pannexin (Panx-1) channels, as demonstrated by blocking activity or expression of channels, and inactivating extracellular ATP. By monitoring dye-spreading into adjacent cells, we show that HSP70 released from human monocytes in response to macrophage colony-stimulating factor, prevents the formation of GJIC between monocytes and HMEC. Therapeutic manipulation of this pathway could be of interest in inflammatory and tumor growth.

INTRODUCTION

Heat shock protein 70 (HSP70) which was initially described as an intracellular protein [1-3] is also released into the circulation under various stress conditions [4-9]. Circulating HSP70 is increased in pathological conditions including cancer cell invasiveness and metastasis [10-12]. Irrespective of whether HSP70 enters the circulation via an active or passive release mechanism, the role of this extracellular HSP70 remains poorly understood.

High levels of circulating HSP70 are reported to correlate with monocyte adhesion to endothelial cells [13]. Among the systems that mediate cell-to-cell interaction, gap junctional intercellular communication (GJIC) is an important modulatory factor for growth, migration and differentiation of cells. Gap junctions are plasma membrane domains containing intercellular channels that allow a direct exchange of ions and small molecules between adjacent cells. The channels are composed of two hemichannels and are formed when a hemichannel from one cell docks with a symmetrically opposed hemichannel from a neighboring cell [14]. Each hemichannel is an oligomer of six proteins named connexins (Cx), which form the central pore of the channel. Connexins expressed within the vasculature include Cx37, 40, 43 and 45 [15, 16]. Although they are differentially expressed along the vascular tree, Cx43 is the most widely and highly expressed protein in all cell models and human tissues. The permeability of gap junctions can be affected by a number of mechanisms, including changes in cytosolic

ion concentrations and Cx43 phosphorylation [17]. Their aberrant function has been associated with a number of pathological conditions, including cancer and inflammation [18-21].

Whereas the role of Cx hemichannels formed by Cx43 in modulation of monocytes-endothelial adhesion is well identified [22, 23], the role of the paracrine intercellular communication is less clear. This paracrine communication does not require cell-cell apposition, as it involves release of one or more signalling molecules into the extracellular medium, and their subsequent interaction with receptors, such as G protein-coupled receptors (GPCRs), on neighboring cells [24]. One of these paracrine factors is adenosine triphosphate (ATP), which is released through unpaired Cx hemichannels (not connecting cells) and propagates intercellular Ca^{2+} waves.

Extracellular HSP70 induces signal transduction through the LPS receptor CD14 in monocytes and macrophages [25, 26]. In malignant cells, HSP70 also induces a TLR4-dependent EGFR phosphorylation, which triggers MAPK signaling [6, 25, 27]. Because LPS or EGF induce Cx43 phosphorylation leading to GJIC abrogation [28, 29], we examined whether extracellular HSP70, exogenously added or released from circulating monocytes, modulates GJIC and Cx43-hemichannel functions in human microvascular endothelial cells (HMEC) via the engagement of specific membrane receptor-associated signaling pathways in HMEC.

RESULTS

Extracellular recombinant human HSP70 (rhHSP70) blocks the gap junction intercellular communication (GJIC) between human microvascular endothelial cells (HMEC)

The effects of rhHSP70 were analyzed on the functionality of gap junctions established between HMEC in confluent monolayer by using the gap-FRAP technique [30, 35]. Briefly, HMEC were loaded with a diffusible tracer (calcein/AM), and the fluorescence of investigated cells was suppressed by a laser beam. The recovery of fluorescence in these cells, which results from the intercellular diffusion of calcein from neighboring cells, was recorded to measure the diffusion rate constant k (min^{-1}), an index of gap junction permeability. Figure 1A shows typical changes in the fluorescence of cells, before and after photobleaching. rhHSP70 was used at 5 µg/ml (Fig. 1B, open circles), a concentration that evoked both maximal spreading of HMEC spheroids (Suppl. Fig. S1) and increase in cell motility (Suppl. Fig. S2). rhHSP70 prevented the fluorescence recovery normally observed in cells exposed to control solution (black circles), demonstrating that rhHSP70 blocked the GJIC between HMEC (Fig. 1B). A time-dependent decrease in k was observed within 1 h (from 0.417 ± 0.100 min^{-1} in untreated to 0.032 ± 0.014 min^{-1} in rhHSP70-treated cells; mean ± SD, n=8; Fig. 1C). In comparison, HMEC exposure to rhHSP27 rather increased k (from 0.488 ± 0.207 min^{-1} in untreated cells to 0.643 ± 0.277 min^{-1}; n=8). Addition of polymyxin B (PMB100 ng/ml) did not prevent the time-dependent decrease in k value observed with rhHSP70 (from 0.445 ± 0.111 min^{-1} in control to 0.097 ± 0.100 min^{-1}; n=3). Thus, the rhHSP70-induced GJIC inhibition is mainly caused by the rhHSP70 protein itself, rather than by any contaminating endotoxin.

Extracellular rhHSP70 modulates Cx43 protein expression and phosphorylation

Connexin 43 (Cx43) which is the most widely and highly expressed gap junction protein [36], is detected at the level of gap junction plaques and within the intracellular space of HMEC cultures (Fig. 2A). Consistent with GJIC abrogation, rhHSP70 decreased Cx43 at the plasma membrane within 30 min and disrupted the Cx43 gap junction plaques within 1h. As Cx43 incorporated into gap junction plaques is insoluble in Triton X-100 [32], we subjected HMEC to a Triton X-100 fractionation assay and determined the relative amount of Cx43 in the junctional plaques. Figure 2B shows that rhHSP70 provoked a drastic reduction in Cx43 expression at the plasma membrane (46 ± 6% of control after 1 h; $^{**}P<0.001$, n=5). We did not detect significant changes in expression of the other endothelial-specific Cx37 and Cx40 (Suppl. Fig. S3).

Specific serine phosphorylations in the C-terminal tail of Cx43 [37] were increased by rhHSP70 within 1 h (Fig. 2C), as expected for a blockage of GJIC [38, 39]. All these phosphorylating effects of rhHSP70 were antagonized by cell pretreatment with a neutralizing antibody against toll-like receptors (TLR) 4 (*Ab*TLR4) (Fig. 2D).

Zonula occludens 1 (ZO-1) is the major protein that interacts with Cx43, precisely through its C-terminal region, to form functional gap junction plaques [40, 41]. Interestingly, inhibition of ZO-1/Cx43 interaction has been shown to promote Cx43 phosphorylation on Ser368. As shown in Figure 2E, HMEC displayed large cell border-localized ZO-1 which was not delocalized upon 1 h of rhHSP70 application. Furthermore ZO-1 coimmunoprecipitated with Cx43 in control as well after 30 min of cell exposure to rhHSP70 but not in the following time periods (Fig. 2F). Disruption of the Cx43/ZO-1 interaction coincides not only with the reduction of Cx43 present at the plasma membrane, but also its phosphorylation at Ser368, as previously reported [42] (but not of Tyr265, Suppl. Fig. S3).

Extracellular rhHSP70 mediates EGFR transactivation contributing to GJIC abrogation

Heat shock and exogenous HSP70 were shown to activate toll-like receptors (TLR) 2 and 4, as well as to promote their association with the epidermal growth factor receptor (EGFR) and the receptor phosphorylation [6]. Since TLR2 is not detected in HMEC, we hypothesized that rhHSP70 may transactivate EGFR through its interaction with TLR4. Accordingly, the rhHSP70-induced EGFR phosphorylation was prevented by the neutralizing *Ab*TLR4 antibody while being unaffected by PMB (Fig. 3A).

After boiling rhHSP70 solution for 30 min, which denaturizes the protein but not LPS, EGFR phosphorylation was no longer observed, indicating that the effect of intact rhHSP70 was due to HSP70 itself (Fig. 3B) and there was no active endotoxin in solutions. Pre-treatment of HMEC with AG490 (50µM), a kinase inhibitor of JAK2 and EGFR, partially prevented EGFR phosphorylation (by 45%; n=6; P<0.01).

By adding an HA motif at its N-terminus, we produced a tagged rhHSP70 that was used to distinguish the exogenous from the endogenous proteins. The purified rhHSP70-HA molecule was a single 70-kDa protein as much functional as the commercially available

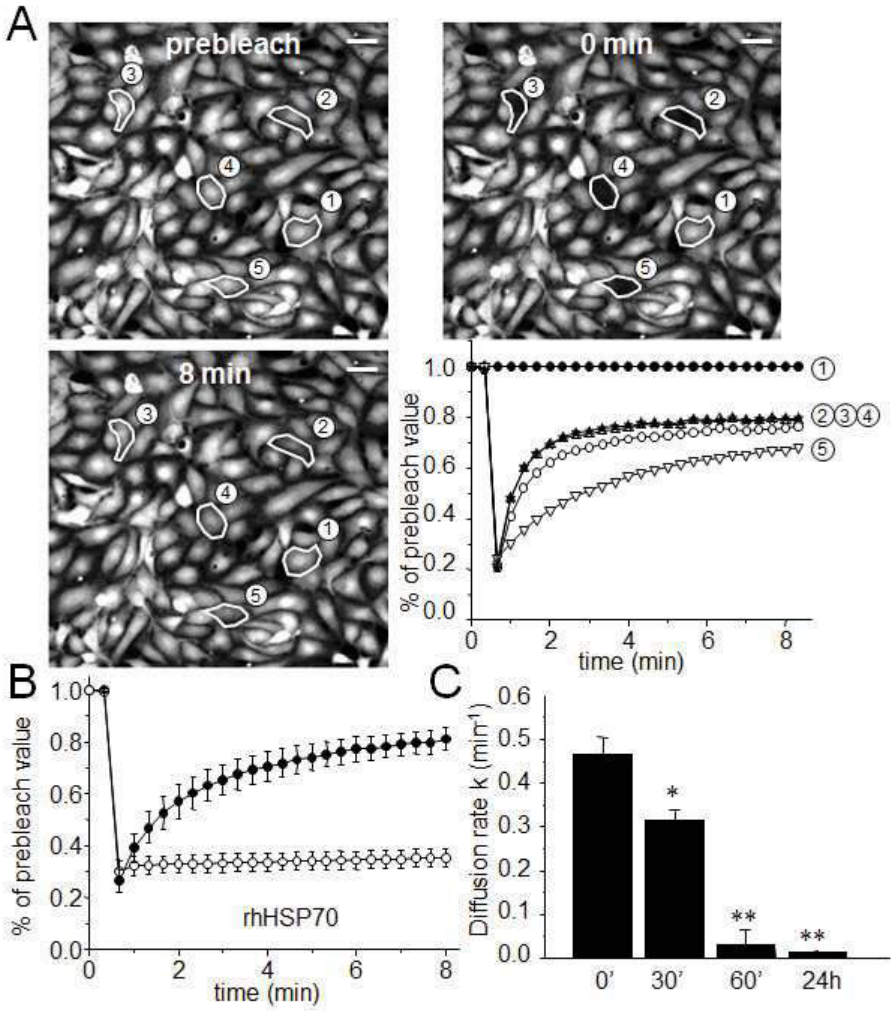

Fig 1: Extracellular rhHSP70 inhibits the endothelial gap-junction coupling. A. FRAP analysis of cell-to-cell communication. Digital images of fluorescence distribution in a HMEC monolayer at three times during a typical gap-FRAP experiment: prebleach, just after bleaching (0 min) and after fluorescence recovery (8 min). Polygons 2-5 are bleached cells, and polygon 1 is an unbleached control cell used for correction of the artefactual loss of fluorescence. Bars 20 µm. Corresponding fluorescence intensities (% of prebleach value) versus time in tested cells. Note the fluorescence recovery follows an exponential time course when the bleached cells are interconnected by open gap-junction channels to unbleached cells (polygons 2-5). The relative permeability of gaps is given by the time constant k. B. Graph represents mean ± SEM of the fluorescence redistribution after photobleaching in coupled HMEC in control (●) or after 60 min (○) with rhHSP70 (5 µg/ml). (C) Histogram shows k values measured after rhHSP70 addition for 0, 30, 60 min and 24 h (mean ± SD, n=8; **P<0.01, *P<0.05 *vs* control [t=0 min]).

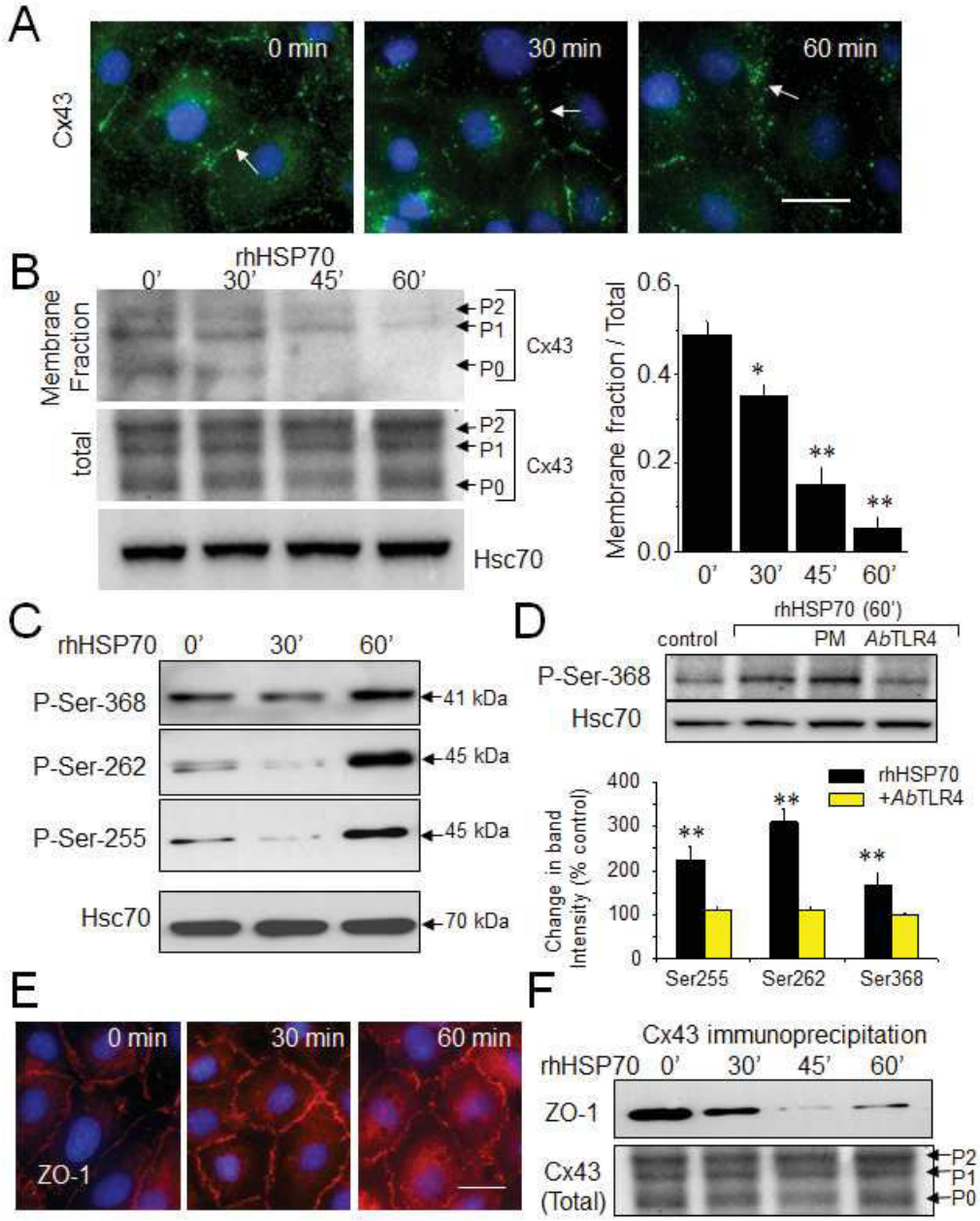

Fig 2: Extracellular rhHSP70 modules membrane level and phosphorylation of Cx43. A. Immunofluorescence detection of Cx43 (green) in HMEC after treatment with 5µg/ml rhHSP70 for indicated times (DAPI staining of nuclei). Arrows indicate Cx43 plaques. Representative of 5 experiments. Bar 20 µm. B. Western blot of the total and membrane fraction (Triton X-100 insoluble) of Cx43. P0, P1 and P2 denotes the three major Cx43 migration bands. Cell membrane lysates immunoblotted for Cx43, after treatment with rhHSP70 for time periods as indicated (Hsc70 as loading control). Right panel shows changes in band intensity of the membrane fraction related to the total Cx43 expression level (mean ± SD, n=5; **P<0.01, *P<0.05 *vs* control [t=0 min] in all cases). C. Effect of rhHSP70 on Cx43 phosphorylation pattern. Western blots using three different antibodies against the carboxy terminal part of Cx43 to detect phosphorylation on serine at position Ser262, Ser255 and Ser368 (representative of 5 experiments). D. rhHSP70 leads to phosphorylate Cx43 in a TLR4-dependent manner. Western blot showing phosphorylation on Ser368. When indicated, cells were pre-treated for 60 min with polymyxin B (PMB10 µM) or the neutralysing anti-TLR4 (*Ab*TLR4 10 µg/ml). rhHSP70 was (or not) added for 60 min (representative of 5 experiments). Histogram shows changes in the phosphorylated status of Cx43 in response to 60 min of cell treatment with rhHSP70 (black) or rhHSP70 plus AbTLR4 (grey), expressed as percentage of control (mean ± SD, n=5; **P<0.01, *P<0.05 *vs* control). E. Immunofluorescence detection of ZO-1 in HMEC after exposure to rhHSP70 for indicated times. Representative of 5 experiments. Cell nuclei stained with DAPI. Bar 20 µm. F. Coimmunoprecipitation of Cx43 and ZO-1 in HMEC, stimulated or not by rhHSP70 for time periods as indicated. The total Cx43 shows slight variations in the unphosphorylated form P0 and the phosphorylated forms P1 and P2 (Hsc70 as loading control; representative of 4 experiments).

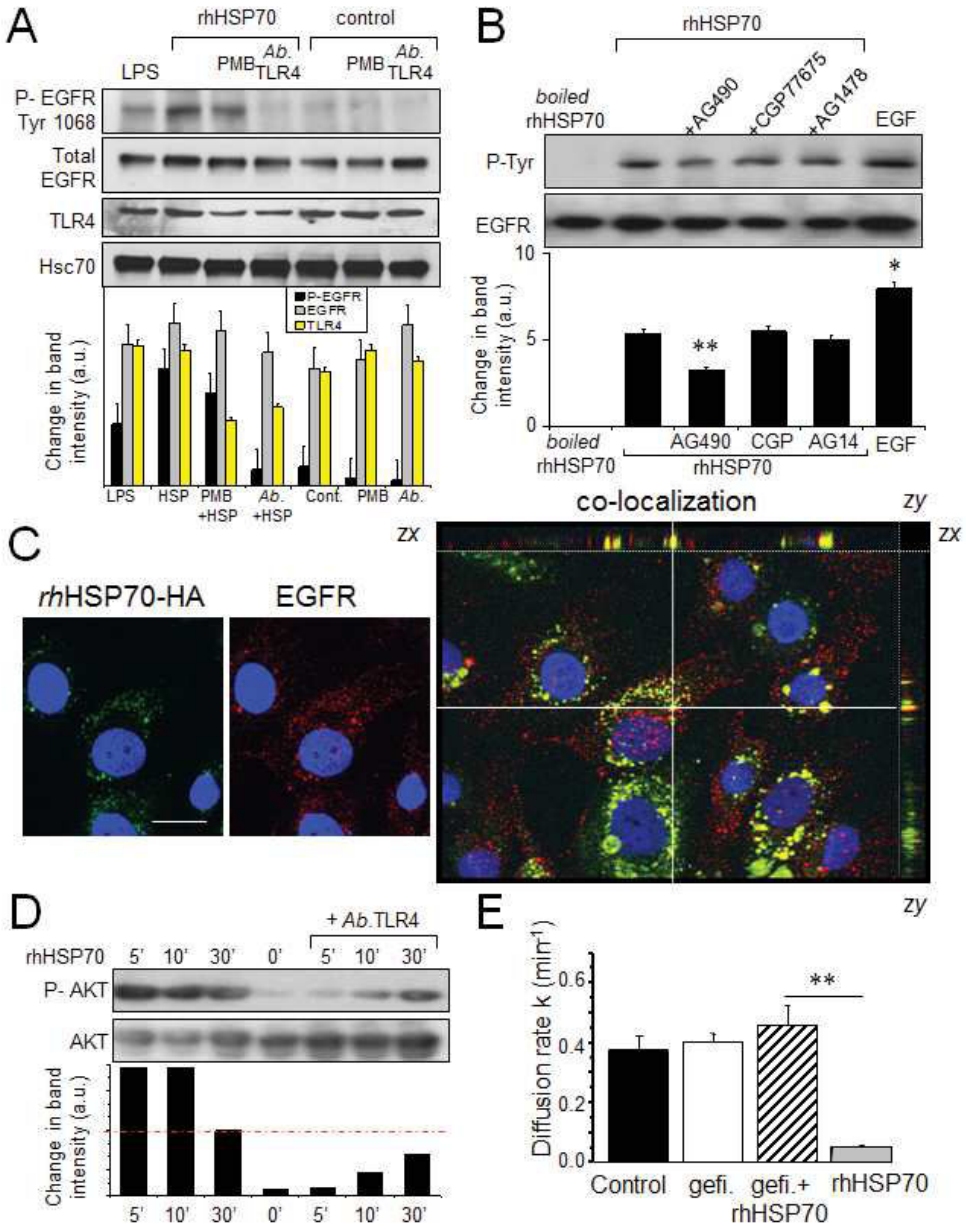

Fig 3: Extracellular rhHSP70 induces a TLR4-dependent EGFR transactivation leading to the GJIC abrogation. A. Western blot analysis of EGFR Tyr-1068 phosphorylation and TLR4 expression in HMEC, unstimulated (control) or stimulated with rhHSP70 or LPS (1 µg/ml) for 15 min. When indicated, cells were pre-treated for 60 min with polymyxin B (PMB10 µM) or the neutralising anti-TLR4 (*Ab*TLR4 10 µg/ml). Lower panel shows changes in the band intensity (mean ± SD, n=3; Hsc70 as loading control). B. Tyrosine phosphorylation of EGFR by rhHSP70 involves the kinase JAK2. Western blot analysis of EGFR phosphotyrosine (P-Tyr) after EGFR immunoprecipitation in HMEC. Cell pretreatment with the kinase inhibitors AG1478 (AG14; 5µM), CGP77675 (CGP; 1µM), AG490 (50µM) for 30 min before exposure to rhHSP70 or 100 ng/ml EGF for 15 min. A boiled rhHSP70 (100°C, 30 min) known to denaturize protein but not LPS, was used to evaluate the contribution of contaminants to the EGFR activation. Lower panel shows changes in band intensity (mean ± SD, n=5; **P<0.01, *P<0.05 *vs* rhHSP70 [t = 0 min] with AG490). C. *P*artial co-localization of rhHSP70 and EGFR. HMEC were stimulated with rhHSP70-HA for 5 min and double-stained for EGFR (ErbB1). Representative micrographs and corresponding cross-sections (xz and yz) showing a three-dimensional stack of rhHSP70 (green), EGFR (red) and the combined image of co-localization (yellow); DAPI staining of nuclei. Optical section of 0.5 µm thickness (n=3, bar 20 µm). D. Phosphorylation of AKT. Cells were exposed to rhHSP70 for the indicated time periods, and lysates were immunoblotted using antibodies recognizing phosphorylated or total forms of AKT. When indicated, cells were pretreated for 60 min with the neutralising anti-TLR4 (*Ab*TLR4 10 µg/ml). Lower panel shows corresponding changes in the band intensity. E. Contribution of EGFR signaling to rhHSP70-induced GJIC inhibition. Diffusion rate constants k determined from recovery curves for HMEC after 1 h in control, Gefitinib (Gefi; 10µM) or Gefi plus rhHSP70 (mean ± SD, n=4; **P<0.01, *P<0.05 vs control).

recombinant molecule (Suppl. Fig. S4). rhHSP70-HA was observed to be internalized into serum-starved HMEC within 5 minutes, and to partially co-localize with EGFR (Fig. 3C).

The AKT activation, which is crucial to disrupt GJIC by causing phosphorylation of Ser368 in Cx43 [43, 44], was rapidly induced by rhHSP70 (within 5 min) in a TLR4-dependent manner (Fig. 3D). The EGFR tyrosine kinase inhibitor, gefitinib (10μM) also antagonized the inhibitory effect of rhHSP70 on GJIC (Fig. 3E). Altogether, our data suggest that rhHSP70 transactivates EGFR.

Extracellular rhHSP70 induces intracellular Ca^{2+} mobilization

Since EGFR engagement activates a calcium-dependent signaling [45, 46], we measured the 340/380 nm ratio of fura-2 fluorescence, reflecting the cytosolic $[Ca^{2+}]i$ in PM-pretreated, fura-2-loaded HMEC (Fig. 4A, upper trace). In nominally Ca^{2+}-free bath conditions, rhHSP70 induced a transient increase in $[Ca^{2+}]i$ within 1–3 min, which was followed by three or more Ca^{2+} waves. These oscillations, never spontaneously observed

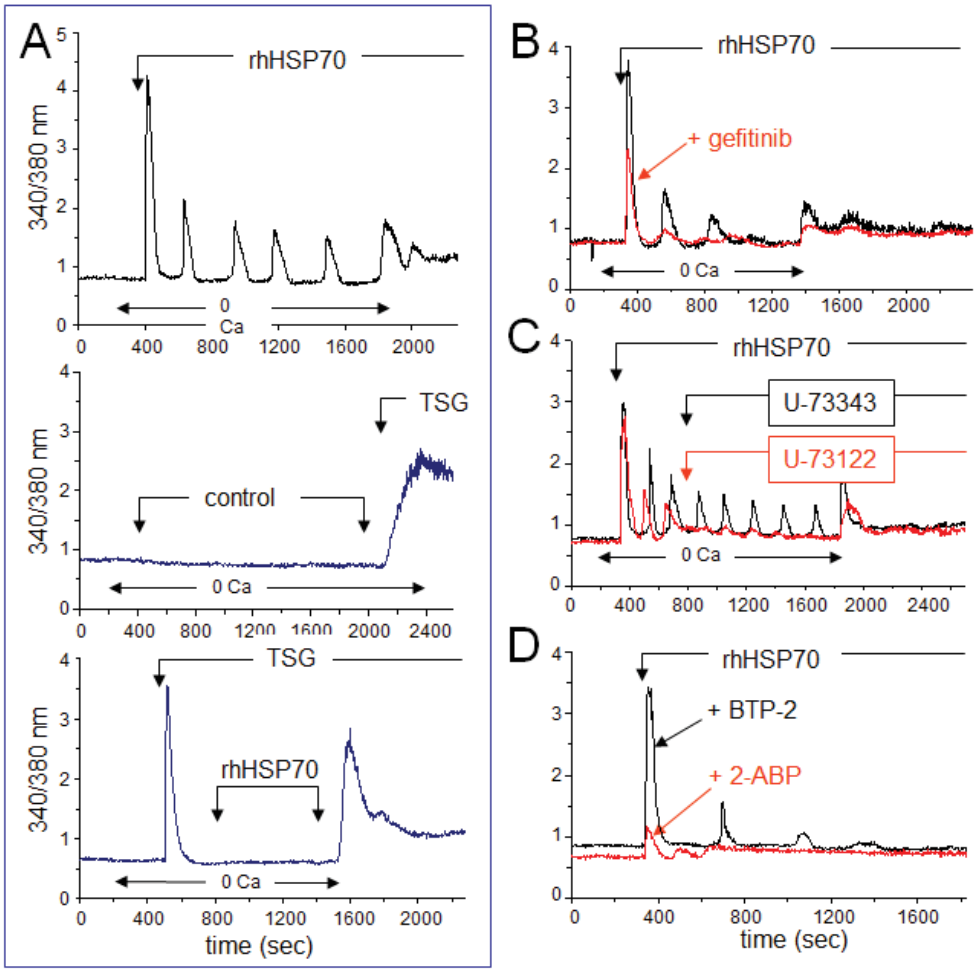

Fig 4: Extracellular rhHSP70 induces intracellular Ca^{2+} mobilization. A. rhHSP70 induced Ca^{2+} release from internal stores in HMEC. Data are expressed as the 340/380 nm excitation ratio in one cell to observe oscillations in $[Ca^{2+}]i$ because the oscillatory process is not synchronized in cells of the same monolayer. External additions of drugs are indicated by arrows. Changes in external calcium bath conditions are indicated on the bottom of traces. In most cases, drugs were initially applied in the absence (0 Ca) then in Ca^{2+} (1.8 mM) containing solution to reveal Ca^{2+} release from internal stores then external Ca^{2+} entry, respectively (representative from 50 cells; n=10). No calcium increase was induced by the cell superfusion of the control bath solution (middle trace) while thapsigargin (TSG 4μM) always produced a drastic increase in $[Ca^{2+}]i$ (lower trace; Representative from 50 cells; n=4). B. Contribution of EGFR to rhHSP70-induced Ca^{2+} signaling. Superimposed traces from cells preincubated with the EGFR (ErbB1) inhibitor, gefitinib (10 μM for 30 min; in red), before the addition of rhHSP70 (Representative from 50 cells; n=5). C. Effects of phospholipase C (PLC) inhibitor U-73122 (5 μM) and its inactive analog U-73343 (5 μM) on the rhHSP70-induced Ca^{2+} oscillations. Drugs were applied without (0 Ca) then with extracellular Ca^{2+} (1.8 mM) (representative from 50 cells; n=5). D. Ca^{2+} oscillations required both Ca^{2+} release from internal stores and store operated Ca^{2+} entry (SOCE). Cells were pretreated with the selective SOCE inhibitor, BTP-2 (20 μM; 20 min), before challenged with rhHSP70 (Representative from 30 cells; n=4).

in control (middle trace), could be prevented by the selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (4μM), suggesting that rhHSP70 recruits Ca^{2+} from intracellular stores (lower trace). Furthermore, rhHSP70 induced phosphorylation of AKT, ERK and SAPK/JNK within 5–10 min, which was significantly attenuated by the Ca^{2+} chelator BAPTA/AM (Suppl. Fig. S5).

The EGFR tyrosine kinase inhibitor, gefitinib (10 μM), attenuated the Ca^{2+} signal elicited by rhHSP70, i.e. the initial peak amplitude was decreased at 56 ± 3% of the control amplitude (n=20; $P<0.05$; Fig. 4B). These results demonstrate that an amplification loop involving intracellular calcium and EGFR activation mediates the effects of rhHSP70 in HMEC.

EGFR transactivation is known to stimulate phospholipase C (PLC), leading to inositol 1,4,5-trisphosphate (InsP3) formation and release of Ca^{2+} from InsP3-sensitive Ca^{2+}-stores. The extracellular application of U-73122 (5 μM), an inhibitor of PLC, reduced both the number and amplitude of rhHSP70-evoked Ca^{2+} oscillations (Fig. 4C) [47]. In contrast, the same dose of its inactive analog, U-73343, had no effect. Furthermore, the cell pretreatment with BTP-2 (20 μM), a cell-permeable blocker of store-operated Ca^{2+} entry (SOCE), decreased both frequency and amplitude of Ca^{2+} oscillations without affecting the initial peak of Ca^{2+} release and the basal $[Ca^{2+}]i$ (Fig. 4D). Pretreatment with 2-APB (50 μM), a blocker of InsP3 receptors [48], decreased basal $[Ca^{2+}]i$ and suppressed Ca^{2+} oscillations. Thus, intracellular Ca^{2+} oscillations evoked by rhHSP70 are related to the initial release of Ca^{2+} from InsP3-sensitive intracellular Ca^{2+}-stores.

Ca^{2+} mobilization and GJIC blockage are partially dependent on ATP release by HMEC

Extracellular ATP inactivation with apyrase (20 U/ml) significantly reduced both the frequency and amplitude of $[Ca^{2+}]i$ oscillations while keeping fairly conserved the amplitude of the initial Ca^{2+} peak (Fig. 5A). Moreover, apyrase partially antagonized the blocking action of rhHSP70 on GJIC (Fig. 5B). Cell exposure to rhHSP70 induced a significant release of ATP as demonstrated using the luciferin-luciferase bioluminescence assay (Fig. 5C).

Given that rhHSP70-induced cytosolic Ca^{2+} oscillations in HMEC depend, at least in part, on the release of ATP and subsequent P2 purinergic receptor activation, we supposed that Cx43 hemichannels could act as a putative pathway of ATP release. Inhibition of Cx43 channels, either with 18βGA (10 μM, n=2; not shown) or with the mimetic peptide Gap26 (500 μM) totally suppressed the rhHSP70-induced ATP release from HMEC (Fig. 5C) and significantly attenuated rhHSP70-induced Ca^{2+} response, especially the oscillatory process (Fig. 5D). The remaining ATP release, also observed in control cells, was unable to evoke Ca^{2+} oscillations in HMEC (Fig. 5D) and could be blocked by the vesicular transport inhibitor brefeldin A (20 μM; not shown).

Recent evidence emerged indicating that a cross-inhibition of pannexin (Panx) channels, especially Panx-1, by mimetic peptides (Gap26) and carbenoxolone, is involved in ATP release and Ca^{2+} currents in various cell types [49]. Therefore we tested a specific inhibitor of Panx-1, probenecid (Prb), at a dose that does not affect Cx channels [50]. Prb reduced rhHSP70-evoked Ca^{2+} oscillations without affecting the initial peak (Fig. 5E) and totally blocked the rhHSP70-evoked ATP (Fig. 5F). The total inhibition of cytosolic Ca^{2+} oscillations was achieved with Gap26. When the Cx43 expression was reduced by siRNA (Fig. 5F), the rhHSP70-induced ATP release was significantly decreased (by 40%), and further abolished by Prb (Fig. 5F).

HSP70 release from stimulated human monocytes prevents heterocellular GJIC with HMEC

The release of ATP and subsequent activation of endothelial intracellular Ca^{2+} signalling are reported to modulate monocyte adhesion to endothelial cells and their transendothelium migration [23, 51]. Exposure of human, peripheral blood monocytes to M-CSF for 12 hours increased the expression and release of HSP70, without affecting Cx43 expression and phosphorylation (Fig. 6A, B, C). The amount of HSP70 secreted by monocytes seems very low compared with the exogenously added in HMEC cultures. However this was a dosage for the whole fluid bathing the cells whereas the secretion by monocytes must be considered in their closed vicinity near the endothelial cell. So the real quantity of HSP70 secreted by the monocyte and collected by the endothelial cell is certainly much higher that the dose measured (diluted) in the whole bath. Knocking down HSP70 with a specific *si*RNA reduced by about 80% the amount of HSP70 found after 12 h into the bath of M-CSF-treated monocytes (Fig. 6D). M-CSF-stimulated monocytes increased ATP release by HMEC, which could be mediated by HSP70 released from these monocytes (Fig. 6E).

To evaluate the impact of released HSP70 on the establishment of GJIC between monocytes and HMEC, these monocytes were double loaded with calcein, a dye that is able to pass through gap junctions, and with DiL, a membrane-bound stain used to distinguish cell donor (monocyte) from recipient cells (HMEC) (Fig. 6F). Note that calcein is an intracellular dye that becomes fluorescent after hydrolysis by cytosolic esterases [52]. After hydrolysis, calcein is highly charged and therefore impermeable to cell membranes; it is thought to travel from cell to cell through gap junctions [35, 53]. DiL, conversely, does not travel from cell to cell [54]. Preloaded

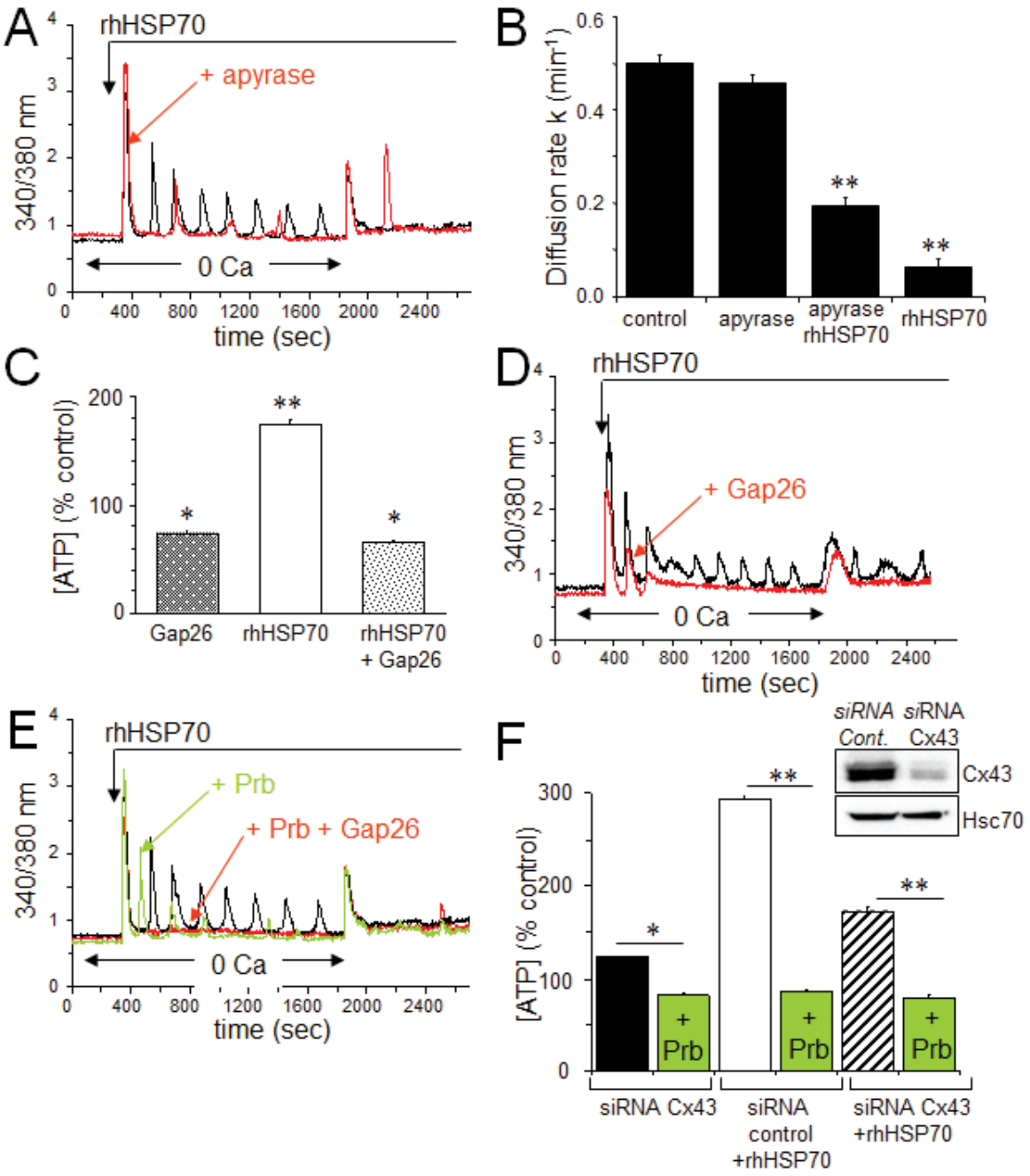

Fig 5: The rhHSP70-induced ATP release contributes to endothelial Ca^{2+} oscillations. A. Contribution of the ATP release to the rhHSP70-evoked Ca^{2+} oscillations in HMEC. Cells were either untreated (black) or pretreated for 30 min with 20 U/ml apyrase (red) before to be exposed for 60 min to rhHSP70 (representative of 20 cells; n=5). B. Apyrase partially antagonizes the inhibitory effect of rhHSP70 on the GJIC between HMEC pretreated by apyrase then apyrase plus rhHSP27. Histogram shows the diffusion k constant measured after 60 min of cell treatments (mean ± SD, n=4; *P*-values<0.05 *vs* control). C. rhHSP70-induced ATP release from HMEC is blocked by Gap26 (500 µM). Extracellular ATP was measured by Luciferase assay (means ± S.D., n=3, *P*-values<0.05 *vs* control). D. Contribution of Gap26-sensitive channels to the rhHSP70-induced Ca^{2+} oscillations. Cells pretreated with *Gap26* (500 µM for 30 min; red) before rhHSP70 (representative of 20 cells; n=5). E. Pannexin-1 modulates the Ca^{2+} oscillatory response to rhHSP70. Superimposed traces obtained from cells stimulated with rhHSP70 in the presence or absence of the Panx-1 blocker, 100 µM probecenid (Prb; green), or Prb plus Gap26 (red) (Representative of 10 cells; n=5). F. siRNA Cx43 knockdown attenuates the rhHSP70-induced ATP release. HMEC were transfected with Cx43 and control siRNA 48 h prior to various analyses. Insert is representative western blot showing the specific depletion of Cx43. Histogram shows the amounts of ATP released (relative to control cells) in response to rhHSP70 (1h). In some cases, transfected cells were exposed to 100 µM Prb (mean values ± SD, n=5; **P<0.01, *P<0.05 vs control).

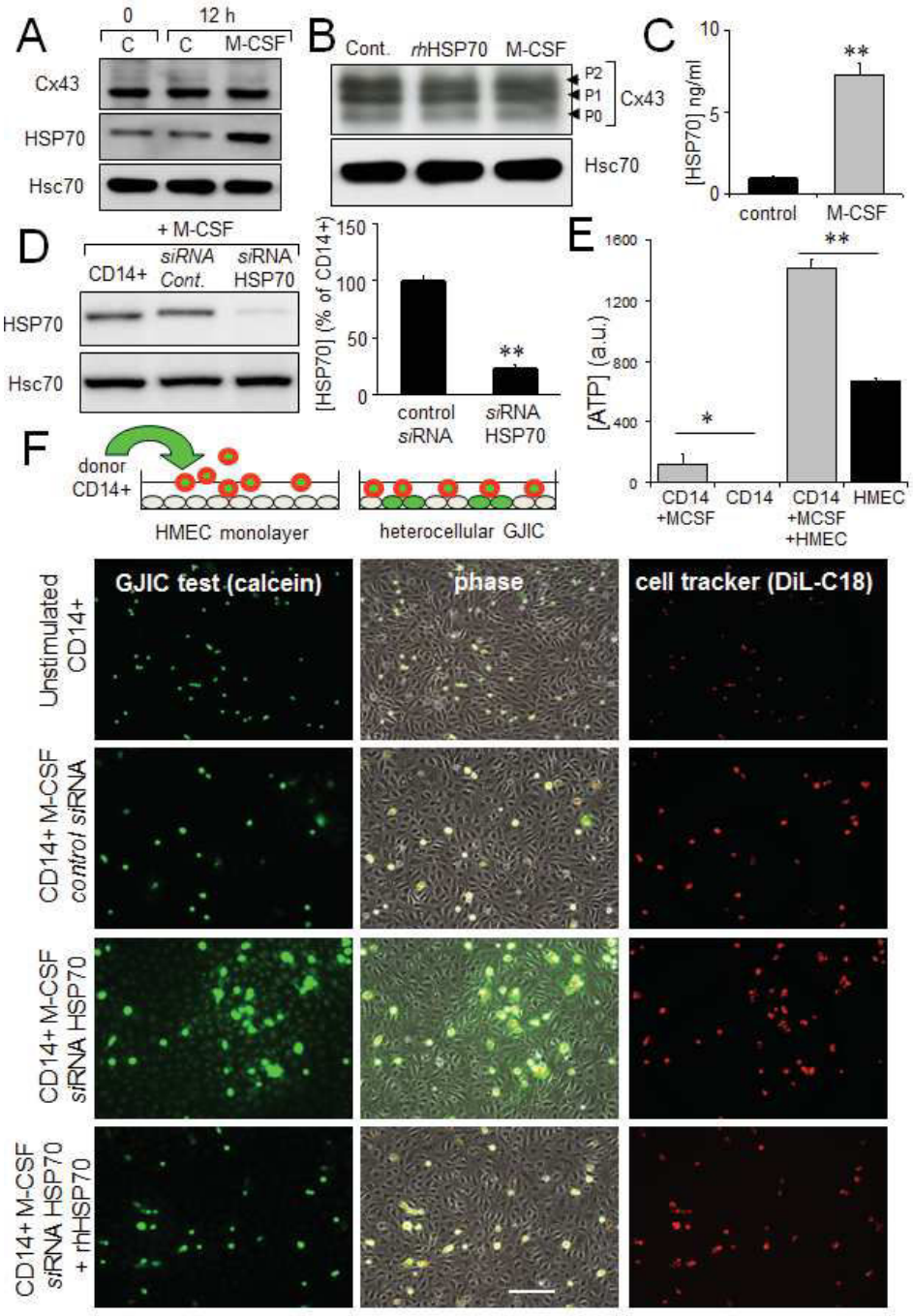

Fig 6: The HSP70 release by monocytes alters their coupling with HMEC. A. M-CSF (100 nM) increases HSP70 expression in monocytes (representative of 5 experiments; Hsc70 as loading control). B. Cx43 expression in monocytes is not affected by 12h-treatment with M-CSF or rhHSP70 (representative of 3 experiments). C. M-CSF induced HSP70 release. Amounts of HSP70 measured by ELISA in supernatant of monocytes untreated (control) or treated with 100 nM M-CSF for 12 h (mean ± SD; n=4; **P-values <0.01). D. siRNA HSP70 knockdown. Cultured monocytes were transfected with HSP70 or control siRNA 48h prior to various analysis. Left, western blot analysis of protein extracts from cells treated with M-CSF for 12 h. Right, histogram shows HSP70 release by transfected monocytes in response to 100 nM M-CSF for 12 h (mean ± S.D., n=4; representative of 4 experiments). E. The ATP release by HMEC/monocyte cocultures is mainly due to HMEC (bioluminescence assay; means ± S.D. n=3; **P-values<0.01, *P-values<0.05 vs control). F. Functional GJIC between monocytes and HMEC. Monocytes (donors) were preloaded with calcein/AM and DiL-C18. Calcein diffuses through gap junctions, while DiL-C18 does not. Labelled monocytes are then plated with unlabeled HMEC monolayer (receivers). HMEC establishing GJIC with monocytes become fluorescent by calcein diffusion. Only siRNA HSP70-transfected monocytes establish GJIC with HMEC and exogenously added rhHSP70 (5µg/ml) improved it. Phase-contrast microphotographs after 3 h of culture (representative of 6 experiments; Bar 100 µm).

monocytes were then plated with unlabeled HMEC monolayer. After 3 hours of co-culture, unstimulated monocytes remained in suspension, whereas M-CSF-treated cells adhered to the endothelial monolayer (Fig. 6F). The HSP70 knock-down by using a specific *si*RNA, promoted calcein transfer from monocyte to endothelial cells, attesting formation of GJIC. The supplementary addition of rhHSP70 to the bath reversed it. The gap-junction blockers, carbenoxolone (200 μM) and 18βGA (500 μM) similarly blocked this heterocellular dye transfer (not shown). These results make clear that released HSP70 prevents cell-to-cell communication between monocytes and HMEC.

Dye coupling was also seen with the non-transfected monocytes pretreated by the adenosine derived inhibitor of HSP70, VER155008 (Fig. 7A), i.e. VER155008-treated cells established functional gap junctions with HMEC in response to M-CSF. VER155008 treatment did not change the high level of HSP70 protein expressed in cells stimulated by M-CSF (Fig. 7B). Treatment of HMEC alone by VER155008 improved the blocking action of rhHSP70 on GJIC (Fig. 7C).

To explore the role of Cx43 in monocyte-endothelial cell interactions, HMEC were transfected with siRNA Cx43 or control siRNA, and intercellular communication was tested 48 h later (Fig. 7D). Control HMEC cultures (transfected with control siRNA) showed extensive dye transfer of calcein from M-CSF-treated siRNA HSP70 monocytes (on average of 7 neighboring cells). The siRNA-mediated knockdown of Cx43 in endothelial cells did not affect the adhesion monocytes, but abolished (reduced by more 90% from control levels) the heterocellular GJIC. Upon stimulation by M-CSF, the transendothelial migration of monocytes in which HSP70 has been decreased by siRNA was strongly increased as compared to control cells (Fig. 7E). This "diapedesis" increase was abolished by knocking down the endothelial Cx43 expression.

DISCUSSION

The major contribution of our study is the demonstration that extracellular HSP70, exogenously added or released from human circulating monocytes in response to M-CSF induction, activates a signaling cascade that decreases GJIC activity between HMEC and HMEC/monocytes reducing monocyte transmigration through the endothelium (Fig. 8). Thereby, HSP70 release may play a regulatory role in monocyte diapedesis when invading tissues to differentiate into macrophages or osteoclasts, depending on tissue microenvironment. Therapeutic manipulation of this pathway would be a strategy in various diseases such as chronic inflammatory diseases, osteoporosis, and cancer.

The protective functions of intracellular HSP70 are well documented whereas the role of extracellular HSP70 has been less explored [8, 11, 55]. Here, we show that extracellular HSP70 specifically decreases expression of Cx43 at the plasma membrane and induces hyper-phosphorylation of Cx43 in a time-dependent manner, leading to GJIC blockage. Since a reduced expression of Cx43 in endothelial cells has been shown to decrease the formation of vessels in Matrigel [19], this decreased expression may explain why rhHSP70 affects the spreading pattern of endothelial cells as compared to the typical sprouting when exposed to VEGF. The decreased expression of Cx43 in response to HSP70 is associated with the phosphorylation of its serine residues, which was shown to regulate gap junction channel formation, permeability, and turnover in other cell types [38]. In addition, rhHSP70 inhibits the ZO-1/Cx43 interaction at the plasma membrane, reducing the amount of Cx43 at the cell surface. Accordingly to Palatinus and coworkers [56], we confirm that inhibition of this interaction favors phosphorylation on Ser368. Specific phosphorylation of Cx43 has also been observed to promote GJIC inhibition in rat microvascular endothelial cells exposed to LPS [28].

HSP70 was observed to physically interact with TLR4, which transactivates EGFR. Inhibition of TLR-4 and its downstream signaling cascade with specific neutralizing antibodies efficiently suppressed HSP70-induced tyrosine phosphorylation of EGFR in HMEC. The use of kinase inhibitors suggested that EGFR transactivation may only slightly depend on intrinsic EGFR tyrosine and Src kinase activities, but may require kinase activity that is partially inhibited by the tyrphostin AG490.

In several cell types including HMEC [57, 58], EGFR activation elicits an initial Ca^{2+} peak that depends on inositol-1,4,5-triphosphate (InsP3) through induction of Ca^{2+} release from intracellular stores, followed by a plateau phase dependent on a secondary Ca^{2+} entry from the extracellular compartment. The oscillatory behavior of secondary Ca^{2+} entry depends on the cell type. Extracellular HSP70 induces such a biphasic Ca^{2+} response in HMEC, *i.e.* an initial peak followed by $[Ca^{2+}]i$ oscillations in nominally Ca^{2+}-free external conditions. As already described in EGF-treated cells [59], the source of Ca^{2+} responsible for these oscillations is the InsP3-sensitive endoplasmic reticulum (ER) store. Due to the modulation of InsP3 receptors by Ca^{2+}, the "pacemarker" elevation which precedes the spikes could be explained by a slowly rising level of $[Ca^{2+}]i$ released by InsP3 until a threshold $[Ca^{2+}]i$ is reached to elicit the rapid upstroke [60, 61]. By using BTP-2, a blocker of store-operated Ca^{2+} entry (SOCE), and 2-APB, a potent blocker of InsP3 receptors, we show intracellular Ca^{2+} oscillations evoked by rhHSP70 are mainly due to the release of Ca^{2+} from InsP3-sensitive Ca^{2+}-stores although the frequency of oscillations require calcium entry across the plasma membrane.

Release of ATP was shown to promote an oscillatory

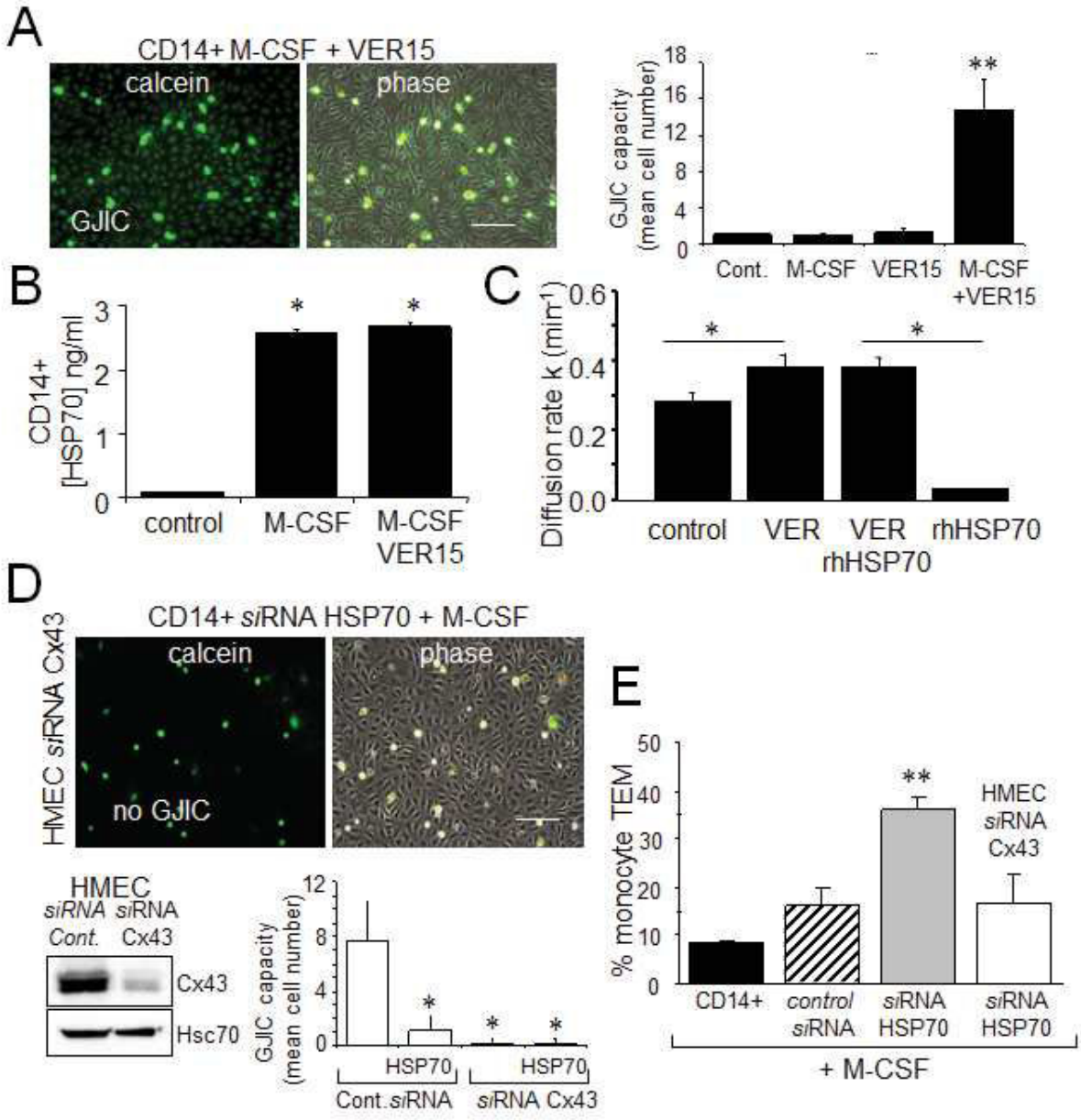

Fig 7: The endothelial Cx43 expression is required for the transendothelial migration of monocytes. A. The adenosine-derived inhibitor of HSP70, VER155008 (10 μM) favors the establishment of GJIC between monocytes (M-CSF stimulated) and HMEC within 3 h. Phase-contrast microphotographs are representative of 4 experiments. Bar 100 μm. Right, histogram represents the total cell number of HMEC receiving dye (calcein) per monocyte (mean ±SD, n=3; **P-values <0.01 vs control). B. VER155008 does not inhibit the HSP70 release by M-CSF-stimulated monocytes for 12 h (dosed by ELISA; mean ± SD, n=4; *P-values <0.05 vs control). C. VER155008 antagonizes the blocking effect of rhHSP70 on GJIC between HMEC (gap-FRAP analysis). Histogram shows the constant k measured for the coupled cells after 1 h of cell treatment (mean ± SD, n=4; *P-values<0.05 vs control). D. Effects of the endothelial Cx43 knockdown on the GJIC coupling between HMEC and HSP70 depleted monocytes. Cultured HMEC and monocytes were transfected respectively with siRNA Cx43 (HMEC) and siRNA HSP70 (monocytes) or control siRNA, 48h prior to various analysis. Insert is representative western blot showing the specific depletion of Cx43 in HMEC. Transfected monocytes (donors), stimulated overnight with M-CSF, were pre-loaded with calcein and DiL-C18 before to be plated. Microphotographs of monocytes in contact with transfected HMEC (receivers) after 3 h of culture (representative of 6 experiments; Bar 100 μm). Histogram represents the mean cell number of neighboring HMEC receiving dye (calcein) per monocyte (mean ±SD; n=50 labeled monocytes examined; n=3). E. Effects of the endothelial Cx43 knockdown and released HSP70 on the transendothelial migration of monocytes. Control or transfected HMEC monolayers grown on Transwells were kept in FCS-free conditions overnight. Control or transfected monocytes ($3x10^5$) stimulated overnight by M-CSF, were labeled with phycoerythrin-conjugated anti-CD14+ before to be added into the wells. Cells migrating through the endothelial layers were counted (after 3 h). Data are percentage of total applied monocytes counted by flow cytometry (mean ± SD; n=5).

increase in cytosolic Ca^{2+} [62] , underlying the paracrine intercellular communication [63-67]. Here we demonstrate that extracellular HSP70 leads to an immediate and robust release of ATP by HMEC. The Cx43 inhibition (Gap26) or knockdown only attenuated the ATP release from HSP70-treated HMEC, suggesting Cx43-hemichannels contribute but are not mainly involved in rhHSP70-induced ATP release. Note that a reduction of Cx43 expression not only lead to a reduction of gap junction function, but also might trigger more complex cellular alterations. In contrast, inhibition (Prb) of Panx-1 function or suppression of its expression (Suppl. Fig. S6) totally abolished rhHSP70-induced ATP release [49, 50, 68]. On the basis of inhibitor data and siRNA experiments, we conclude that Panx-1 channels mainly contribute to the observed rhHSP70-evoked ATP.

Taken together, our experiments indicate that HSP70 released from M-CSF-treated monocytes does not affect monocyte adhesion to endothelial cells but prevents cell-cell communication between monocytes and HMEC. Note that Ca^{2+} chelation did not affect monocyte adhesion but slowed down monocyte transmigration through human microvascular endothelium [51]. The role of gap junctions in endothelial paracellular permeability is not well understood. Rather, adhesion complexes such as tight junctions and adherens junctions are established as regulators of permeability at the membrane, restricting paracellular flux [69]. From our observations, it is unlikely that the gap junctions contribute in adhesive function. Our studies also exclude a significant role of connexins as opening hemi-channels. Therefore, the connexins likely participate in the permeability response to inflammatory stimuli through their function in intercellular GJIC, by facilitating the intercellular exchange of signal that coordinate or enhance the response. In addition to Ca^{2+} ions, micro RNA may also pass through gap junctions, suggesting it as an additional candidate among signaling molecules [70]. Moreover, our data suggest that M-CSF, as secreted by endothelial cells in atherogenesis, contribute to the accumulation of monocytes at the site of inflammation by stimulating HSP70 release and subsequently inhibiting monocytes transmigration. Similar mechanisms could be involved in cancers where monocytes accumulation has deleterious effects [71].

Homocellular and heterocellular cross-talks between monocytes and endothelial cells involve multiple receptor-ligand complexes and ion channels, including gap junctions and their connexin protein building blocks,

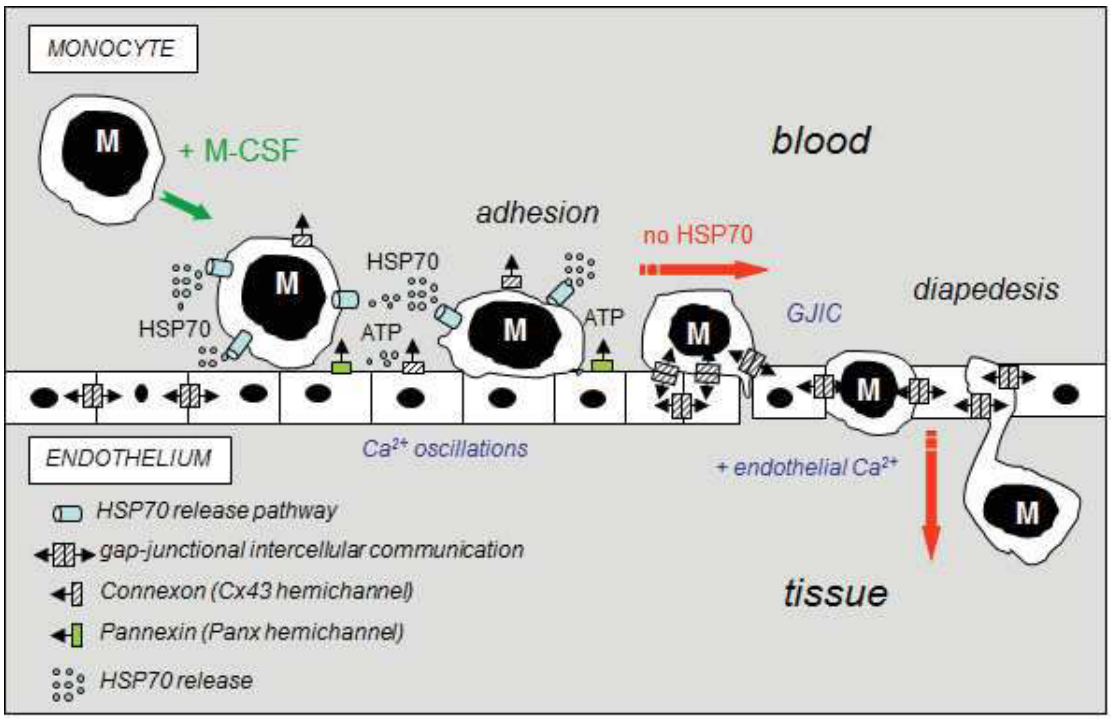

Fig 8: Hypothetical model of the inhibitory effects of extracellular HSP70 on the diapedesis of monocytes. The diagram shows the distribution of gap-junctions (double barrel) and hemichannels (single barrel) that can operate between monocytes (M) and endothelial cells of the microvascular wall. M-CSF-stimulated monocytes adhere to the endothelial cell monolayer and release HSP70 that disrupts GJIC between EC contributing to the subsequent release of ATP through Panx-1 and Cx43 hemichannels from endothelial cells. Extracellular HSP70 induce endothelial Ca^{2+} oscillations without affecting the intercellular tight junction protein, localized between apposed cells. When HSP70 is blocked (siRNA or VER155008), M-CSF-stimulated monocytes communicate with endothelial cells by gap junctions (GJIC), allowing their migration across the endothelial cell monolayer, in an endothelial Ca^{2+}-dependent mechanism (slowed down by BAPTA, a Ca^{2+} chelator, Suppl. Fig. S5).

which have been compared to immunological synapses [72]. By targeting connexins and the channels they form, HSP70 may be part of the complex cascade of molecular interactions that underpin the rolling of monocytes on the endothelial wall. HSP70 interaction with TLR4, Cx43 phosphorylation and decreased expression, ATP release, and Ca^{2+} oscillations, providing several potential targets to therapeutically modulate the transendothelial migration of monocytes.

MATERIALS AND METHODS

Cells

Human microvascular endothelial cells (HMEC; Lonza; Basel, Switzerland) were grown in DMEM plus 10% FCS (5% CO_2; 37°C). Human peripheral blood monocytes were isolated from buffy coats of healthy donors by Ficoll gradient (MACS system, Miltenyi Biotec Inc., Paris, Fr). CD14 monocytes isolated beads were plated in RPMI1640 plus 10% FCS and stimulated overnight by 100 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; Millipore, Molsheim, Fr). Untouched monocytes were nucleoporated with siRNA using Amaxa nucleofector kit (Amaxa; Koln, Germany) and HMEC were transfected by lipofectamine RNAiMAX (Invitrogen; Life Technologies, Saint-Aubin, Fr). siRNA HSP70 was purchased from Sigma-Aldrich (SASI_Hs01_00051449; Saint-Quentin Fallavier, Fr), siRNA Cx43 was from Santa Cruz Biotech (GJA1_human mapping 6q22.31; Clinisciences; Nanterre, Fr) and control siRNA was from Dharmacon (Fermentas; ThermoFischer, Saint-Remy-les-Chevreuses, Fr). Cells were incubated overnight in FCS-free media before use.

Reagents

Low endotoxin rhHSP70 and rhHSP27 were purchased from Enzo Life Sciences (Villeurbanne, Fr) and rabbit anti-HSP70 from ABR (AffinityBioReagent, ThermoFisher, Fr). Mouse anti-Hsc70, rabbit anti-phospho Cx43 (Ser368, Ser255, Ser262 and Tyr265) and probenecid were from Santa Cruz Biotech. Neutralizing anti-TLR4 and mouse anti-EGFR (ErB1) were from Abcam (Cambridge, UK). Mouse anti-TLR4, polymyxin B and gefitinib were from InvivoGen (Toulouse, Fr). Rabbit anti-P-EGFR (Tyr-1068) and antiphospho- and total Akt (Ser473) were from Cell Signaling (Danvers, USA) and mouse anti-Cx43 from Invitrogen. ZO-1 antibody was from Zymed (Invitrogen). DiL-C18, thapsigargin and fura-2/AM were from Molecular Probes. VER155008 and Gap26 (VCYDKSFPISHVR) from Tocris (McKinley, USA). Other chemicals were from Sigma-Aldrich.

Specific cell treatments

To avoid endotoxin contamination of rhHSP, cells were preincubated with polymyxin B (PMB, 10 µM; 30-60 min) and rhHSP70 solutions were also treated with PMB prior to their use. To block TLR4, cells were preincubated with the neutralizing anti-hTLR4 antibody (10 µg/ml; for 30 min).

Fluorescence recovery after photobleaching (FRAP)

The GJIC between HMEC was measured by means of gap-FRAP method [30]. Cells were loaded with 10 ng/µl of calcein/AM for 15 min. The fluorescence of investigated cells was bleached at 405 nm. The recovery of fluorescence was measured at 488 nm every 20 sec for a time period of 8 min. The fluorescence in one unbleached cell was used to correct the artefact loss of fluorescence. The permeability of gap junctions is estimated by the diffusion rate constant k (expressed in min^{-1}) determined from recovery curves as following: $(Fi - Ft)/(Fi – F0)=e^{-kt}$, where Fi, Ft and F0 are intensities before bleaching, at time t and t=0 respectively.

Immunofluorescence and Imaging

Cells were fixed in 4% PAF and permeabilized with 0.1% Triton X-100 [31]. Images were performed using a Leica SP2 RS confocal microscope (Z-series of optical sections from 0.3-0.6 µm intervals; 512x512 pixels; Rueil-Malmaison, Fr). For co-localization, images were taken on Axio Imager 2 (Carl Zeiss GmbH) with an Apotome2 module (Optical sections of 0.5 µm; 512x512; Oberkochen, Germany).

Triton X-100 fractionation

Triton X-100 soluble and insoluble fractions of Cx43 were separated according to VanSlyke and Musil [32]. HMEC were washed in cold PBS, scraped in PBS supplemented with N-ethylmaleide (10 mM), phenylmethylsulfonyl fluoride (1 mM), and sodium orthovanadate (1 mM), and centrifugated for 4 min at 2000g and 4°C. The pellet was resuspended in 400 µl of complete PBS plus protease inhibitor cocktail and phosphatase inhibitor cocktail 1 and 2. The suspension was incubated on ice with 1% Triton X-100 for 40 min. 175 µl of lysate was centrifugated at 100,000g for 50 min at 4°C. The supernatant was resuspended in 175 µl fractioning buffer. The remaining total lysate and the Triton X-100 insoluble fraction were sonicated for 20 s, and protein content was measured by the Bradford assay.

Immunoprecipitation

Briefly, cells were lysed in RIPA buffer, and immunoprecipitation was performed with antibodies, as previously described [31].

Recombinant protein production

Heat Shock 70kDa protein 1B (HSPA1B) [Homo sapiens] modified with HA Tag in Nter (rhHSP70-HA) has been produced by Proteogenix (Oberhausbergen, France). The rhHSP70 cDNA was cloned in pT7-MAT-1 expression vector in *E.coli* with His tag1 in N-terminal position (Cloning strategy: Hind III / Eco RI2). His tag is intended for affinity purification.

Cytosolic Ca^{2+} concentration

Changes in 340/380 nm ratio of Fura-2 fluorescence were used to measure $[Ca^{2+}]i$. Briefly, cells were incubated with 2 µM fura-2-AM (40 min at 37°C), then in HEPES-buffered saline solution (HBSS) as previously described [33]. Measurements were made on Axiovert 40 (Carl Zeiss) with a 20X objective (fluor, 0.75 NA) attached to a dual-excitation spectrofluorimeter (340/380 nm). Emission (510 nm) was collected at a rate of 20 per minute.

ATP measurement

Concentration of ATP in cell media was detected by luciferin-luciferase assay (ENLITEN ATP Assay, Promega; Charbonnieres, Fr). HMEC were plated at 500 × 10^3 cells/cm^2, growth arrested in FCS-free medium and exposed to apyrase (20 U/ml) or GAP26 (500 µM) or probenecid (100 µM) and/or monocytes stimulated or not by M-CSF (100 nM). Supernatants were collected after 1h to 12h, put on ice and centrifuged at 12,000 *g* for 10 min.

Heterocellular GJIC functionality

Monocytes were labeled with 4 µM calcein/AM (30 min) together with 10 µM DiL-C18 as previously detailed [30, 34]. After washing, 10^3 fluorescent cells were laid on HMEC monolayers. The transfer of dye was visualized after a given time at 37°C. To confirm that the calcein transfer was not due to its non-specific up-take, supernatant collected from donors were added to HMEC. No dye uptake by HMEC was found within 24h.

HSP70 ELISA analyses

HSP70 levels in cell supernatants were evaluated using enzyme-linked immunoabsorbent assay (ELISA kit; Enzo Life Sci. ADI-EKS-715) according to the protocol provided.

Transendothelial cell migration

HMEC were cultured on 3-µm membrane pores of Transwell inserts until confluency, then FCS-free overnight (CytoSelectTM, Cell Biolabs; Euromedex, Mundolsheim, Fr). 300 µl of monocytes suspension were added (3x10^5 cells per well). After 3-hours, migrated cells were labeled with phycoerythrin-conjugated anti-CD14+ antibody and counted by flow cytometry.

Statistical analysis

One-way analysis of variance (ANOVA; Statview Software) was used to compare data groups of at least five independent experiments. Stimulated samples were compared to controls by two-tailed, unpaired t-tests. *P values < 0.05 were significant.

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CONFLICTS OF INTERESTS

None declared.

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Primary tumor- and metastasis-derived colon cancer cells differently modulate connexin expression and function in human capillary endothelial cells

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ABSTRACT

A gradual loss of functional gap junction between tumor cells has been reported with colorectal cancer (CRC) progression. Here, we explored if colon cancer cells could also affect gap junctions in blood capillary cells. Human microvascular endothelial cells (HMEC) were cultured with two CRC cell lines established from a unique patient. SW480 cells, derived from the primary tumor, migrate much faster across HMEC monolayer than SW620 cells derived from a metastatic site. The motile SW480 cells highly express and release HSP27 that increases gap junction formation with HMEC. Soluble HSP27 phosphorylates the connexin Cx43 on serine residues and induces its interaction with the oncoprotein 14-3-3, which promotes Cx43 delivery at the plasma membrane. The factors secreted by less motile SW620 cells do not affect Cx43 expression but up-regulate the expression of the connexin Cx32 through an activation of the chemokine receptor CXCR2. In turn, SW620 secreted factors induce tubulogenesis and ATP release. Altogether, cell lines derived from CRC primary tumor and metastasis differentially adapt endothelial cell functions by modulating connexin expression through released mediators.

INTRODUCTION

The outcome of patients who develop a metastatic colorectal carcinoma (CRC) remains poor, emphasizing the need to better understand the mechanisms of disease progression and metastatic dissemination [1-3]. During metastatic dissemination, a cancer cell quits the primary tumor to enter capillaries of the blood system (intravasation), translocates through the bloodstream to capillaries of distant tissues, exits from the bloodstream (extravasation) across the microvascular endothelium, and finally adapts to the foreign microenvironment of these tissues to proliferate and form new tumor foci. In this process, interactions between cancer cells and microvascular endothelial cells are of utmost importance.

A disturbance of gap junction intercellular communication (GJIC) has been involved in both primary tumor formation [4, 5] and progression toward metastasis [6, 7]. Gap junctions are specific cell-to-cell channels formed by integral membrane proteins called connexins (Cx). These junctions play a role in cell growth and differentiation and in tissue homeostasis [8, 9]. In addition to forming channels that enable a direct exchange of ions and small molecules between cells, Cx are involved in transcription regulation [10, 11]. The most widely studied Cx is connexin 43 (Cx43) [12], which is frequently down-regulated in human tumors, *e.g.* Cx43 loss was associated with cancer progression [13]. The redistribution of Cx from the plasma membrane to intracellular compartments is another feature of cancer

cells, as described for Cx32 and Cx43 during CRC development [11].

The effects of CRC cells on Cx expressed in endothelial cells is less known. Here, we explore how CRC cells modulate Cx-expression and function in endothelial cells. For that purpose, we use two human CRC cell lines established from the same patient [14]. SW480 cell line was established from the primary tumor whereas SW620 was derived from a metastatic site [15]. We show that the culture medium of these cell lines have distinct effects on human microvascular endothelial cells. SW480 cells secrete the small heat shock protein HSP27 (also called HSPB1) that promotes Cx43 phosphorylation and the formation of intercellular gap junctions with HMEC. SW620 cells secrete interleukin-8 (IL-8) and promote receptor CXCR2 expression in HMEC; in turn, CXCR2 increases Cx32 expression and induces ATP release. Such distinct effects could account for the differential ability of cancer cells to migrate through the endothelium, to form metastases and to develop new tumor foci in distant organs.

RESULTS

HSP27 favors communication between endothelial and cancer cells

We first confirmed previous reports [16, 17] showing that the small heat shock protein HSP27 was more expressed in, and secreted by, primary tumor-derived SW480 cancer cells when compared to their metastasis-derived counterpart SW620 cells (Fig. 1A). We also noticed that SW480 cells moved much faster than SW620 cells across a human endothelial cell monolayer (Fig. 1B). Looking for a link between these two observations, we used a specific siRNA to decrease the expression of HSP27 in the two cell lines, and to suppress its secretion by SW480 cells (Fig. 1C). These cells were subsequently double loaded with calcein, a dye that passes through gap junctions, and DiL, a membrane-bound dye (Fig. 1D; [18]). The labelled CRC cells were co-cultured with unlabeled HMECs for 6 hours. Although both SW480 and SW620 cells adhered to the endothelial monolayer, the calcein transfer attesting the formation of GJIC was observed only with SW480 cells. HSP27 down-regulation did not affect cancer cell adhesion to endothelial cells, but abolished the calcein transfer from SW480 to HMECs. A similar result was obtained by inhibiting HSP27 expression in SW480 cells with the OGX427 antisense oligonucleotide and was partially antagonised by the concomitant addition of recombinant human HSP27 (rhHSP27) to the culture medium (Suppl. Fig. S1). These results suggest that HSP27 secreted by SW480 cells increases the communication between cancer cells and endothelial cells.

Extracellular HSP27 also promotes the communication between endothelial cells

To analyze the effects of rhHSP27 on gap junctions between HMECs in confluent monolayers, we used the gap-FRAP technique [18]. Briefly, HMECs were loaded with a diffusible tracer (calcein/AM) before suppressing their fluorescence with a laser beam, then measuring the fluorescence recovery resulting from the intercellular diffusion of calcein. Fig. 2A shows typical changes in the fluorescence of cell after photobleaching. Addition of rhHSP27 to the culture medium (5 µg/ml [19], open circles) increased the fluorescence recovery after photobleaching when compared to controls (Fig. 2B). The amount of HSP27 secreted by SW480 cells seems very low compared with the exogenously added in HMEC cultures (Suppl. Fig. S1). However this was a dosage for the whole fluid bathing the cells whereas the secretion by SW480 cells must be considered in their closed vicinity near the endothelial cell. Cells must be adherent to establish gap junction channels (the intercellular space ranges between 2 and 4 nm). So the real quantity of HSP27 secreted by the SW480 and collected by the endothelial cell is certainly much higher that the dose measured (diluted) in the whole bath. The diffusion rate constant k (min^{-1}), which is an index of gap junction permeability, increased within 30 min from 0.487 ± 0.042 min^{-1} in untreated cells to 0.719 ± 0.097 min^{-1} in rhHSP27- treated cells (mean ± SD, n = 8), then slowly decreased (0.642 ± 0.066 min^{-1} after 1 hour, Fig. 2C). This effect of rhHSP27 was prevented by pretreating the cells with a neutralizing antibody against Toll-Like Receptor-3 (anti-TLR3 mAb 20 µg/ml) for 1 h (Fig. 2D, left panel; [19]). A similar result was obtained by incubating HMEC with SW480 cell-conditioned medium (SW480-CM; collected after 6 h in culture), i.e. the k value increased in a TLR3-dependent manner (Fig. 2D, right panel). Conversely, LPS (1 µM) decreased k value, an effect prevented by the TLR4 inhibitor OxPAPC (30 µg/ ml) (Fig. 2E). Altogether, these results indicate that soluble HSP27 increases the communication between neighboring cells.

SW480-CM promotes the phosphorylation of Cx43 in endothelial cells

Immunofluorescence analyses detected Cx43 mainly at the surface of SW480 cells and in the cytoplasm of SW620 cells (Fig. 3A). The diffusion of calcein between cells depends on the opening of gap junction channels present at the plasma membrane of adherent cells. Since the formation of functional Cx43 gap junction channels requires connexin phosphorylation [20-22], we performed immunoblot analyses of whole-cell extracts using a rabbit polyclonal antibody that recognizes several forms of the phosphorylated protein [12, 18, 21, 22]. SW480 and

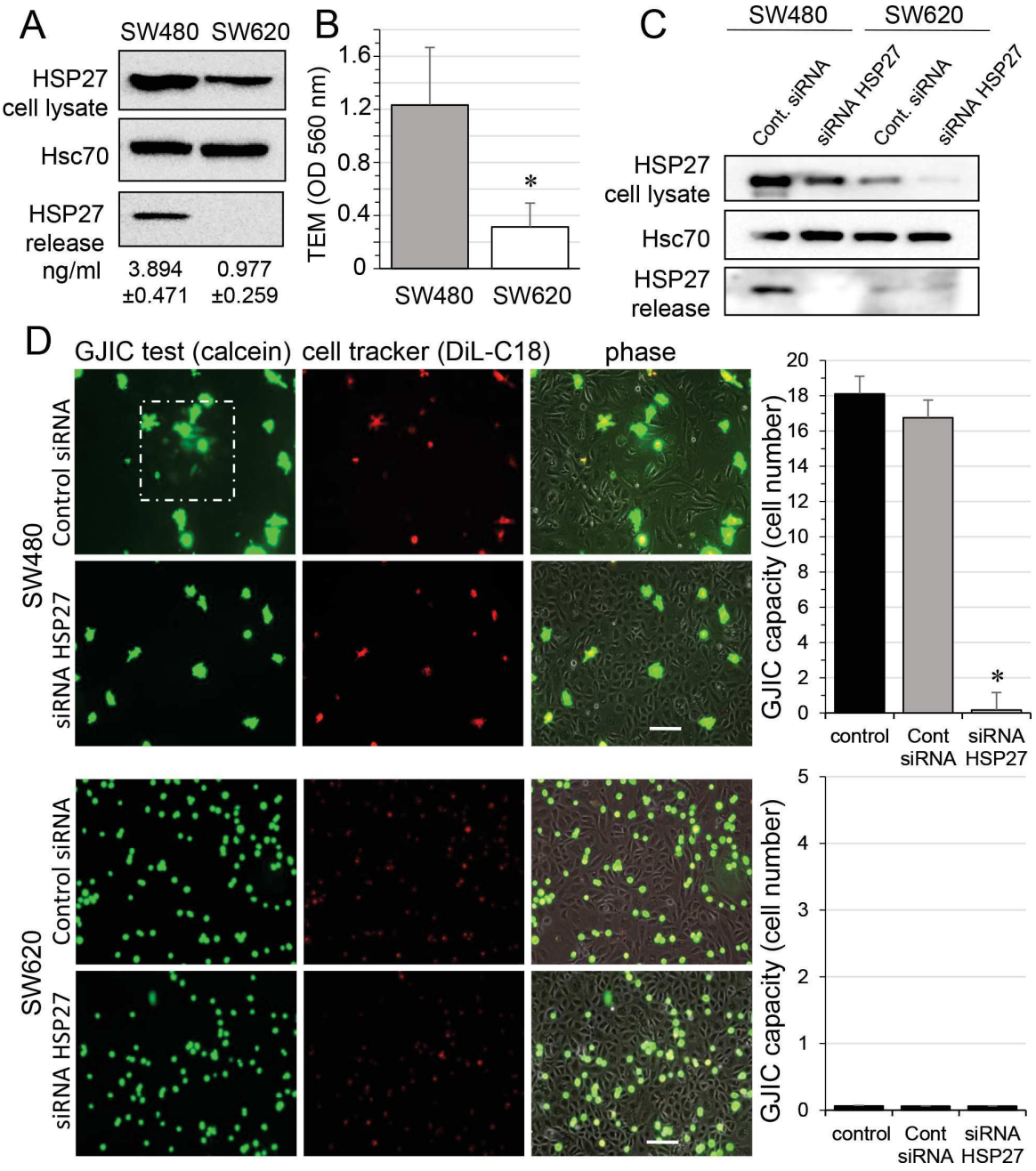

Figure 1: HSP27 knockdown inhibits the gap junctional coupling between SW480 cells and HMEC. A. Expression of HSP27 in the two CRC cell lines, SW480 and SW620 cells. Detectable amounts of HSP27 in supernatants of SW480 cells but not of SW620 cells (media collected after 12 h). Immunoblots representative of 5 experiments (Hsc70 as loading control). Values indicate amounts of HSP27 measured by ELISA in supernatant of SW480 and SW620 cells for 12 h (mean ± SD; $n = 4$; P-values < 0.01). **B.** Transendothelial migration (TEM) of CRC cell lines. Control HMEC monolayers grown on Transwells were kept in FCS-free conditions overnight. Untreated SW480 and SW620 cells (3×10^5) were added into the wells. After coculturing for 6 h, invasive cells on the membrane bottom were stained and quantified at OD 560 nm after extraction (mean ± SD; *P-values < 0.01; $n = 5$). **C.** siRNA transfection decreases the HSP27 expression in CRC cells and suppressed its release by SW480 cells. Representative immune-blot of HSP27 protein level in both SW480 and SW620 cells transfected with control siRNA or siRNA HSP27 for 2 days ($n = 5$; Hsc70 as loading control). **D.** Functional GJIC between SW480 cells and HMEC. The both CRC cell lines, SW480 and SW620 cells (donors), were preloaded with calcein/AM and DiL-C18. Calcein diffuses through gap junctions, while DiL-C18 does not. Labelled CRC cells are then plated with unlabeled HMEC monolayer (receivers). HMEC establishing GJIC with CRC cells become fluorescent by calcein diffusion. Only SW480 cells establish GJIC with HMEC and siRNA HSP27-transfected cells improved it (upper panels). No calcein diffusion was observed from SW620 cells in spite of their adhesion to HMEC (lower panels). Phase-contrast microphotographs after 6 h of culture (representative of 6 experiments; Bar 100 μm). Right, histogram represents the total cell number of HMEC receiving dye (calcein) per CRC cell (mean ±SD, $n = 3$; *P-values < 0.01 vs control).

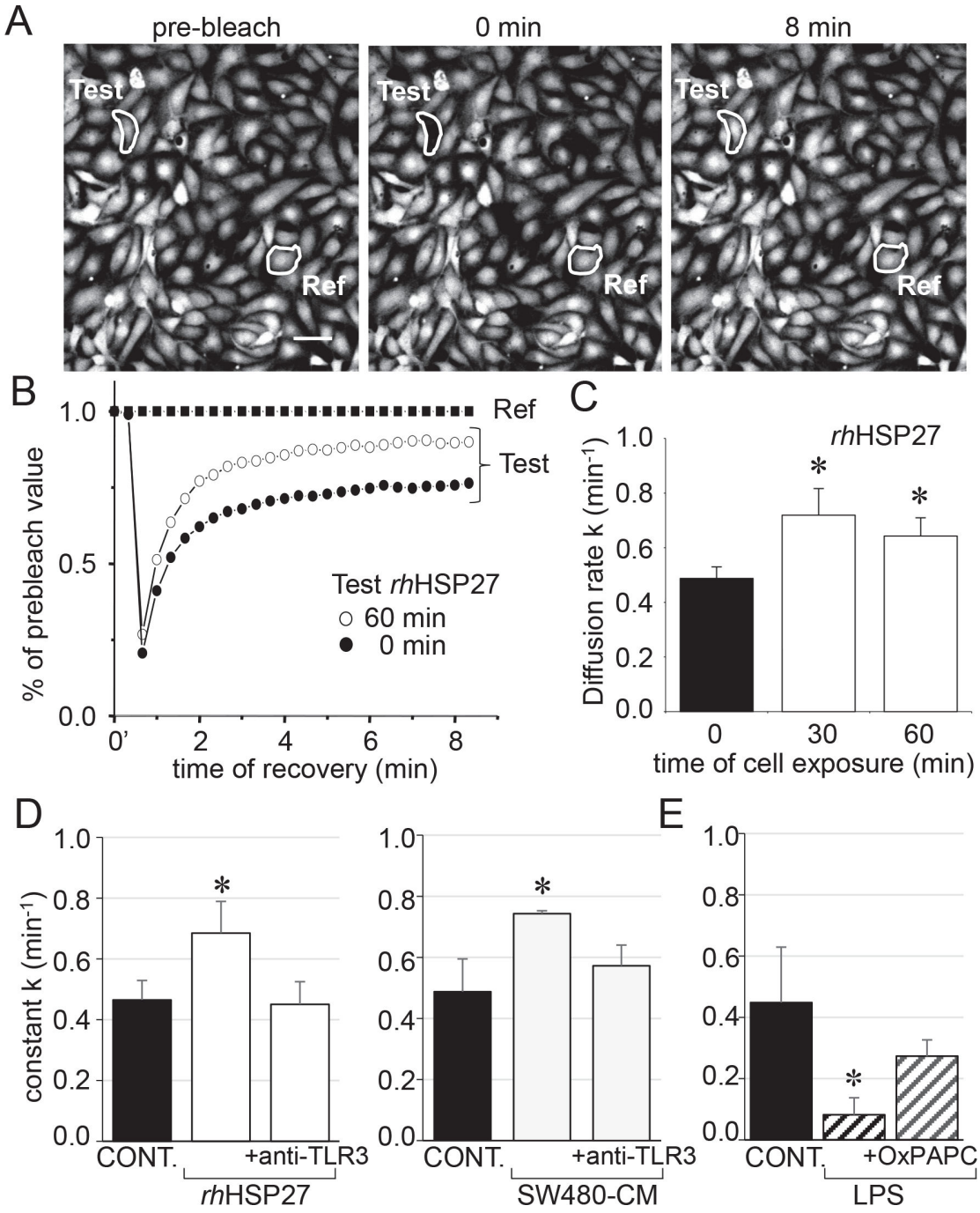

Figure 2: Extracellular HSP27 increases the endothelial gap-junction coupling. A. FRAP analysis of cell-to-cell communication. Digital images of fluorescence distribution in a HMEC monolayer at three times during a typical gap-FRAP experiment: prebleach, just after bleaching (0 min) and after fluorescence recovery (8 min). Bars 20 μm. Corresponding fluorescence intensities (% of prebleach value) versus time in tested cells. The fluorescence in one unbleached cell (Ref) was used to correct the artefact loss of fluorescence. Note the fluorescence recovery follows an exponential time course when the bleached cells (circles) are interconnected by open gap-junction channels to unbleached cells (black squares are Ref). The relative permeability of gaps is given by the time constant *k*. **B.** Recombinant human HSP27 (rhHSP27) effect on GJIC. Graph represents mean ± SEM of the fluorescence redistribution after photobleaching in coupled HMEC in control (●) or after 60 min (O) with rhHSP27 (5 μg/ml). **C.** Histogram shows *k* values measured after the rhHSP27 addition for 0, 30 and 60 min (mean ± SD, $n = 8$; $^*P < 0.05$ *vs* control [$t = 0$ min]). **D.** Both rhHSP27 and SW480-conditioned media (-CM; collected after 6 h) increase the GJIC in a TLR3-dependent manner. Cells exposure for 30 min, in the absence or the presence of neutralizing anti-TLR3 antibody (20 μg/ml) (mean ± SD, $n = 4$; $^*P < 0.01$ *vs* control). **E.** LPS (1 μM) blocks GJIC within 60 min. This inhibitory effect was prevented by OxPAPC (30 μg/ml), a TLR4/TLR2 inhibitor (mean ± SD, $n = 4$; $^*P < 0.01$ *vs* control).

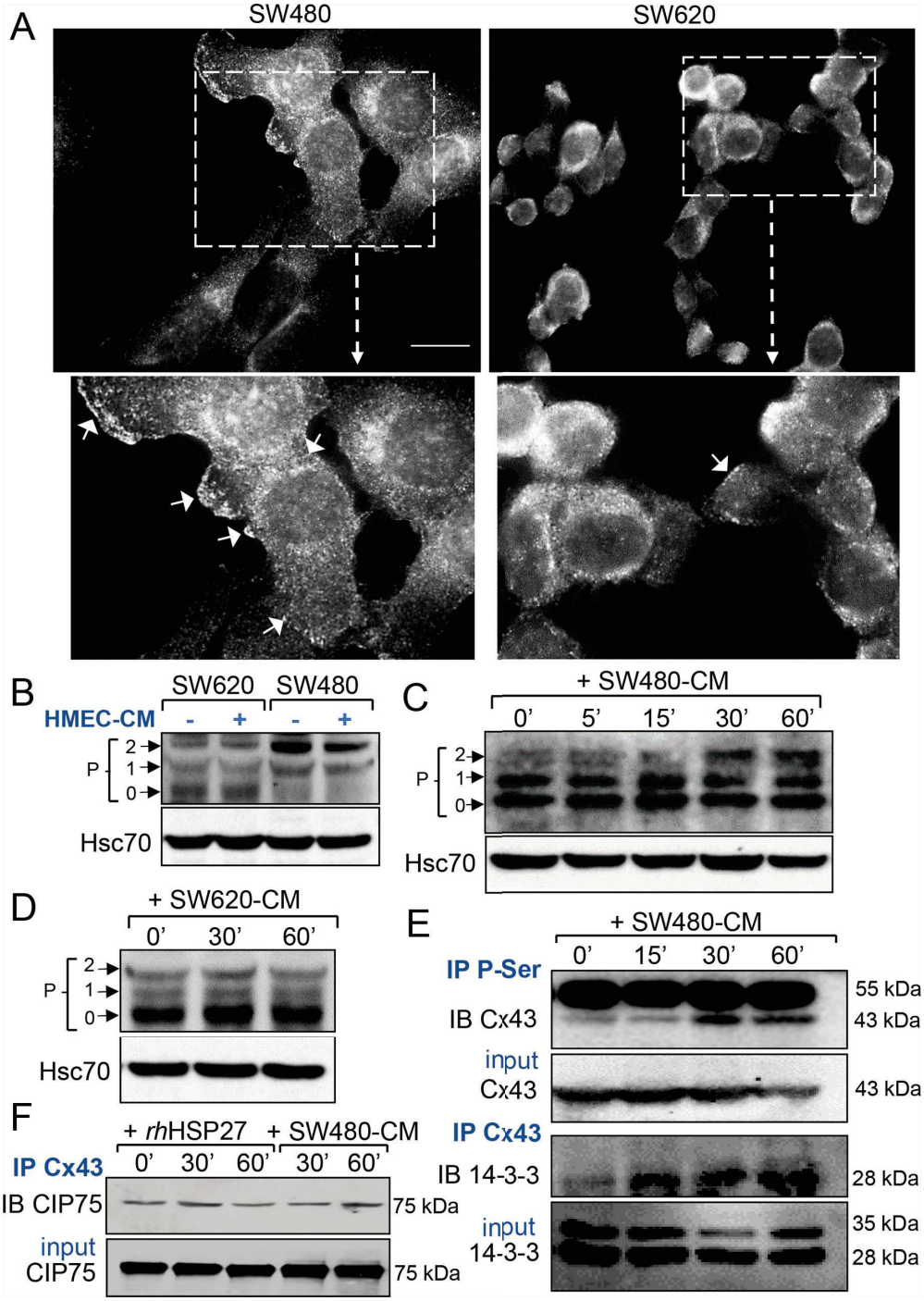

Figure 3: Phosphorylation at serine sites of endothelial Cx43 and 14-3-3 binding characterize the SW480-CM-induced GJIC increase. A. Immunofluorescence detection of Cx43 in SW480 cells and SW620 cells (Bar 20 μm). The dotted areas are enlarged in the inserts below. Arrows indicated the Cx43 plaques at the plasma membrane in cells. Representative of 5 experiments. **B.** Western blot of Cx43 in whole cell lysates from SW480 and SW620 cells, exposed or not the HMEC-conditioned media (-CM, collected after 6 h). P0, P1 and P2 denote the three major Cx43 migration bands (Hsc70 as loading control). **C.** Time-dependent increase in Cx43 phosphorylation induced by the SW480-CM in confluent HMEC. Whole cell lysates in HMEC exposed to SW480-CM (collected after 6 h) for time periods as indicated (Hsc70 as loading control; representative of 3 experiments). **D.** No change in the phosphorylation state of the endothelial Cx43 was induced by the SW620-CM ($n = 3$). **E.** Serine phosphorylation and Cx43 immuno-precipitate in HMEC exposed to SW480-CM. Note that the Cx43 interaction with the protein 14–3-3 precedes its phosphorylation in serine sites. Data are representative of 3 independent experiments. IP, immunoprecipitation; IB, immunoblot; Input material, total amount of proteins per lane. IgG heavy chain at 55 kDa. **F.** Neither rhHSP27 nor SW480-CM affect the low amount of CIP75 interacting with Cx43 (representative of 3 experiments).

SW620 cells expressed distinct patterns of Cx43 (Fig. 3B). SW480 cells expressed mainly a phosphorylated form of Cx43 (called P2 on Fig. 3B), as confirmed by immunoblot treatment with alkaline phosphatase (Suppl. Fig. S2A), whereas SW620 cells expressed mostly the unphosphorylated protein (called P0 on Fig. 3B). Addition of HMEC-CM did not have any effect on the pattern of Cx43 expression in these two cancer cell lines (Fig. 3B and Suppl. Fig. S2A). In confluent endothelial cells, Cx43 was detected mainly as P0 and P1 forms. Incubation of these cells with SW480-CM induced the expression of the phosphorylated P2 isoform (Fig. 3C and Suppl. Fig. S2), which was not observed when HMECs were cultured with SW620-CM (Fig. 3D). The phosphorylation status of Cx43 in HMEC is further demonstrated in Suppl. Fig. S2. Immunoprecipitation of serine-phosphorylated proteins followed by immunoblotting with an anti-Cx43 antibody demonstrated that Cx43 was phosphorylated on serine residues in HMECs upon incubation with SW480-CM (Fig. 3E, upper panels). Looking for the consequences of Cx43 phosphorylation, we immunoprecipitated Cx43, then looked for interaction either with 14-3-3, which was shown to regulate the assembly of Cx43 multimers and their incorporation into existing gap junctional plaques [23, 24], or with CIP75 (Ubiquitine-like-Ubiquitine-associated protein), which regulates Cx43 proteolytic degradation [25, 26]. Incubation of HMECs with SW480-CM promoted the recruitment of 14-3-3 to Cx43 (Fig. 3E, lower panels) while having no effect on Cx43 interaction with CIP75 (Fig. 3F). Of not, rhHSP27 addition to HMEC culture medium also failed to increase Cx43 interaction with CIP75 (Fig. 3F). Moreover, we did not detect a specific ubiquitination of Cx43 in the tested conditions (Suppl. Fig. S2C). Thus, SW480-CM or rhHSP27 did not target Cx43 for proteasomal degradation. Altogether, our results suggest that SW480-CM induces the phosphorylation of Cx43 on serine residues and the subsequent binding of 14-3-3, enhancing the GJIC between cells [23, 24].

SW620-CM induces the expression of a functional Cx32 hemi-channel in endothelial cells

Immunofluorescence analyses revealed that unstimulated HMEC expressed very low levels of Cx32 (not shown) and that the protein was only weakly expressed at the apical membrane of some cells after 6 h of exposure to SW480-CM (Fig. 4A). In contrast, we detected a strong apical membrane and cytoplasmic expression of Cx32 in HMEC exposed to SW620-CM for 6 hours (Fig. 4A). Immunoblot analysis of cell lysates identified a drastic increase in Cx32 expression in HMEC exposed to SW620-CM (Fig. 4B). Using an *in vitro* matrigel tube formation assay [19], we observed also that SW620-CM could promote the ability of endothelial cells to form capillary-like structures (i.e., increased branches per cell; Fig. 4C, 4D). To explore the contribution of Cx32 to this effect, we performed loss-of-function experiments through intracellular transfer of a Cx32 blocking monoclonal antibody [27]. Cx32 blockade dramatically reduced the ability of HMEC incubated with SW620-CM to form capillary-like structures (Fig. 4C, 4D).

Since Cx32 was not involved in GJIC between cells (Fig. 1D and Suppl. Fig. S2B), we next explored the role of Cx32 hemi-channels in ATP release by endothelial cells [28, 29]. Incubation of endothelial cells for 6 hours with SW480-CM and SW620-CM induced a 5-fold and a 10-fold increase in ATP release, respectively (Fig. 5A). Although pannexin (Panx) channels have been involved in ATP release [30], these proteins might not be responsible for the observed effects as Panx-1 mRNA level dramatically decreased in HMECs exposed to SW480-CM and SW620-CM (Fig. 5B) while Panx-2 or Panx-3 were not expressed in HMECs in our culture conditions (not shown). Immunoblot analysis of Panx-1 in whole cell extracts of HMEC revealed no significant difference ($P = 0.498$) between control cells and those incubated with CRC-CM for 6 h (Fig. 5C). This may be explained by the long half-life of Panx-1 (more than 8 hours; [31]) which contrasts with the rapid turnover of Cx43 (with a short life-time of only 1-3 hours; [32, 33]). Nevertheless, cell surface localization of Panx-1 was strongly reduced by SW620-CM as seen by confocal microscopy (Fig. 5D). It is therefore unlikely that Panx-1 channels are responsible for the ATP release increased by SW620-CM. The gap junction blocker carbenoxolone completely blocked SW620-CM-induced ATP release, which was also dramatically reduced by the neutralizing anti-Cx32 mAb (Fig. 5E). Thus, one of the consequences of Cx32 expression increase in endothelial cells exposed to SW620-CM is the release of larger amounts of ATP.

SW620 cell-secreted factors induce the endothelial Cx32 expression and tube formation via the cytokine receptor CXCR2

Looking for the secreted factor that may account for the ability of SW620-CM to promote Cx32 expression in endothelial cells, we explored the production of interleukin-8 (IL-8) as metastastic tumor cells can release high levels of this cytokine [34, 35]. Accordingly, SW620 secreted much more IL-8 than SW480 cells, a secretion that was only slightly increased by incubation with HMEC-CM (Fig. 6A). Since IL-8 interacts with the G-protein-coupled receptor CXCR1 and CXCR2, we explored the expression of these receptors in endothelial cells. Unstimulated HMEC expressed no CXCR1 (not shown; [36]) and low levels of CXCR2 (Fig. 6B). After a 6 hour exposure to SW620-CM, CXCR1 remained undetected (not shown) whereas the expression of CXCR2 was increased

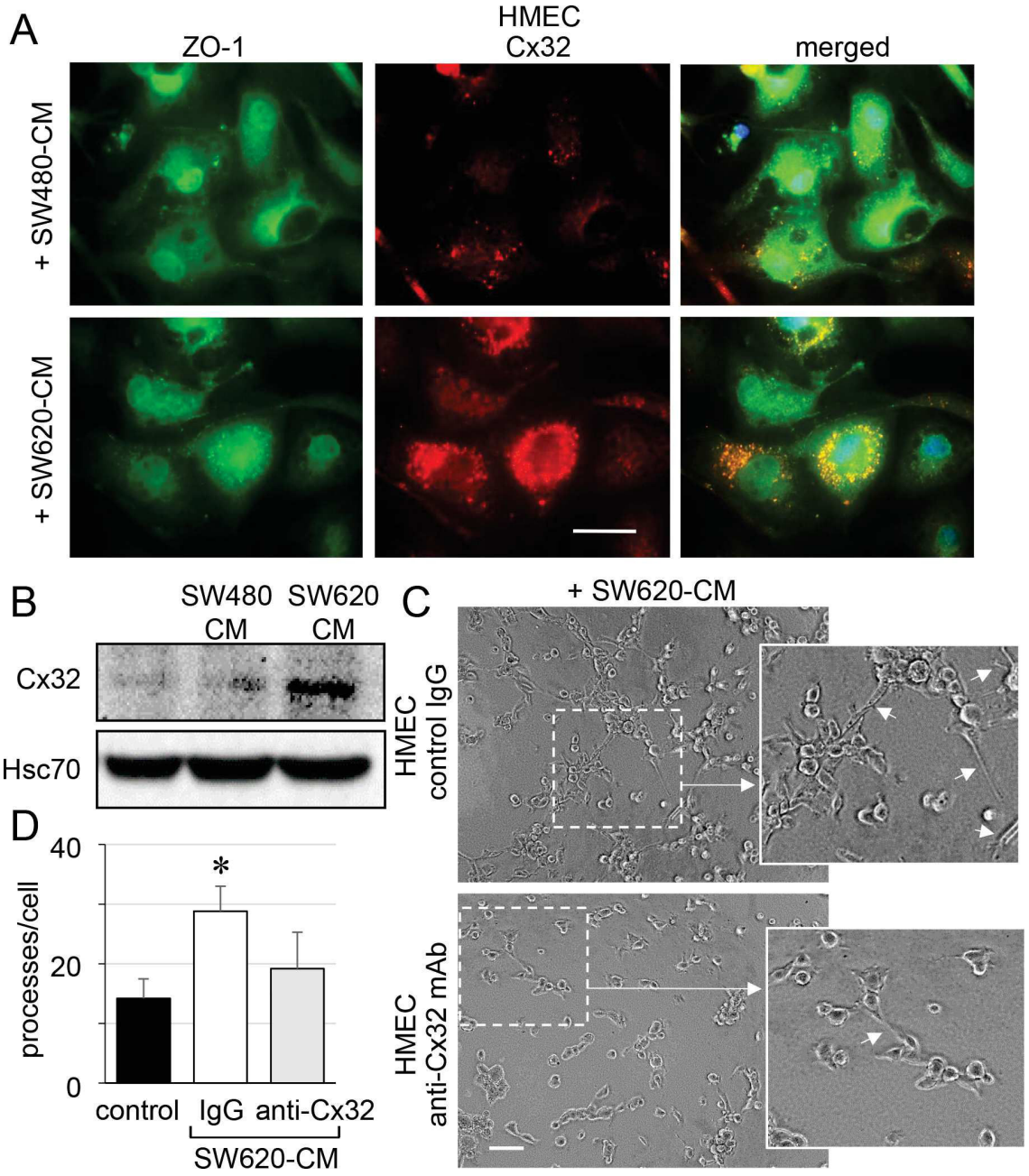

Figure 4: SW620 cell-secreted factors overexpress the endothelial Cx32 favoring tubulogenesis. A. Endothelial cell localization of Cx32 in CRC cell-conditioned media. HMEC were stimulated with SW480-CM or SW620-CM for 6 h and double-stained for ZO-1 and Cx32. Representative micrographs showing the strong labelling of Cx32 induced by SW620-CM and the combined image of co-localization with ZO-1 (yellow); DAPI staining of nuclei (n = 3, bar 20 µm). **B.** SW620-CM increase the Cx32 expression in HMEC. A higher Cx32 protein level was detected in response to SW620-CM compared with SW480-CM by immune-blot analysis (no cell expression in unstimulated HMEC). Representative of 5 experiments (Hsc70 as loading control; 150 µg/lane). **C-D.** *In vitro* tubulogenesis assay of HMEC pretreated or not (control IgG) with inhibitory monoclonal antibody against Cx32 (anti-Cx32 mAb). HMEC were plated on Matrigel-coated 24-well plates, incubated with SW620-CM for 6 h, and photographed. **C.** Representative photos of tube formation in HMEC intracellularly delivered with 0.2 µg anti-Cx32 mAb or control IgG (Bar 80 µm). The dotted areas are enlarged in the inserts on the right. Arrows indicated branch points. **D.** Number of branch points per field of view was quantified (at least 80 single cells were scored; mean ± SD, n = 4; *P < 0.01 *vs* control).

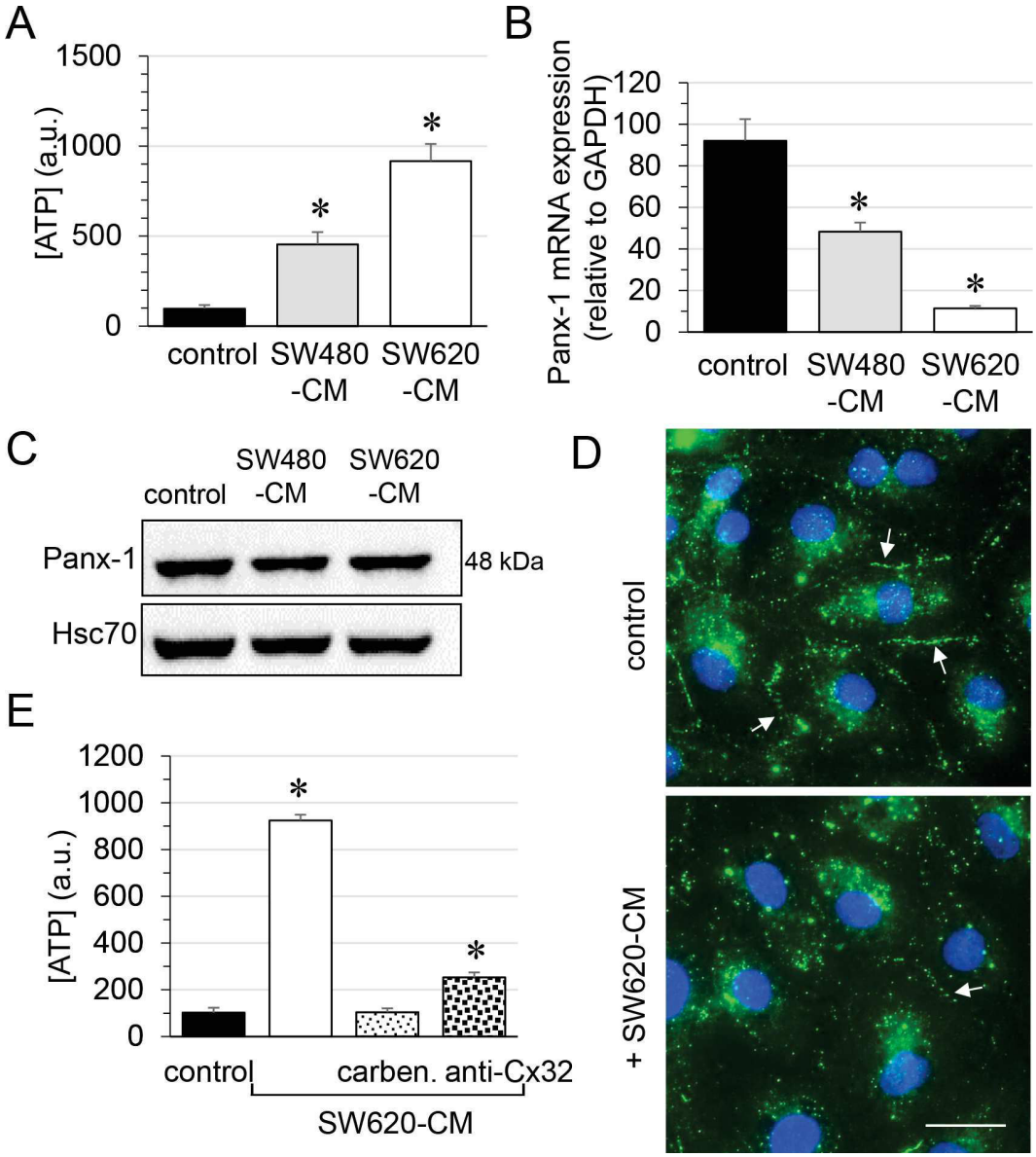

Figure 5: Role of endothelial Cx32 in SW620-CM-triggered ATP release in HMEC. A. Both SW480 and SW620 cell-conditioned media triggered ATP accumulation in HMEC bath medium within 6 h. Extracellular ATP was measured by Luciferase assay (means ± S.D. $n = 4$; **P*-values < 0.01 *vs* control). **B.** Panx-1 mRNA expression in HMEC after 6 h of control or CRC cell-CM exposures. A drastic decrease in Panx-1 expression was observed with SW620-CM (means ± S.D. $n = 3$; **P*-values < 0.05 *vs* control). **C.** Panx-1 protein expression in HMEC was unchanged by exposure to SW480- and SW-620-CM for 6 h (mean ±SD, *P*-values = 0.4980 Mann-Whitney U test; $n = 4$). **D.** Cell surface localization of Panx-1 was decreased in HMEC exposed to SW620-CM for 6 h. Arrows indicated Panx-1 plaques at the plasma membrane. DAPI staining of nuclei. Optical section of 0.5 µm thickness ($n = 5$, Bar 12 µm). **E.** SW620-CM-triggered ATP release is inhibited by gap junction blocker, carbenoxolone (carben., 100 µM, 30 min) and by neutralizing Cx32 antibody (0.2 µg anti-Cx32 mAb) in HMEC (means ± S.D. **P*-values < 0.01 *vs* control; $n = 3$).

(Fig. 6B). Pre-incubation of HMEC with a neutralizing anti-CXCR2 antibody or the CXCR2 inhibitor SB225002 [37] abolished the ability of SW620-CM (Fig. 6C) or IL-8 (Fig. 6D) to promote the formation of tubes by endothelial cells. Both the neutralizing anti-CXCR2 antibody and the CXCR2 inhibitor SB225002 attenuated Cx32 expression induced in HMEC by incubation with SW620-CM (Fig. 6E). In addition to IL-8, we show for the first time that IL-7 could be a potent angiogenic factor that induces tubulogenesis in a Cx32-dependent manner (Fig. 7). Pre-incubation with the Cx32 blocking antibody abolished the ability of IL-7 to promote the tube formation by HMEC. Altogether, SW620-CM induces both CXCR2 expression and function which promotes tubulogenesis, at least in part in a Cx32-dependent manner.

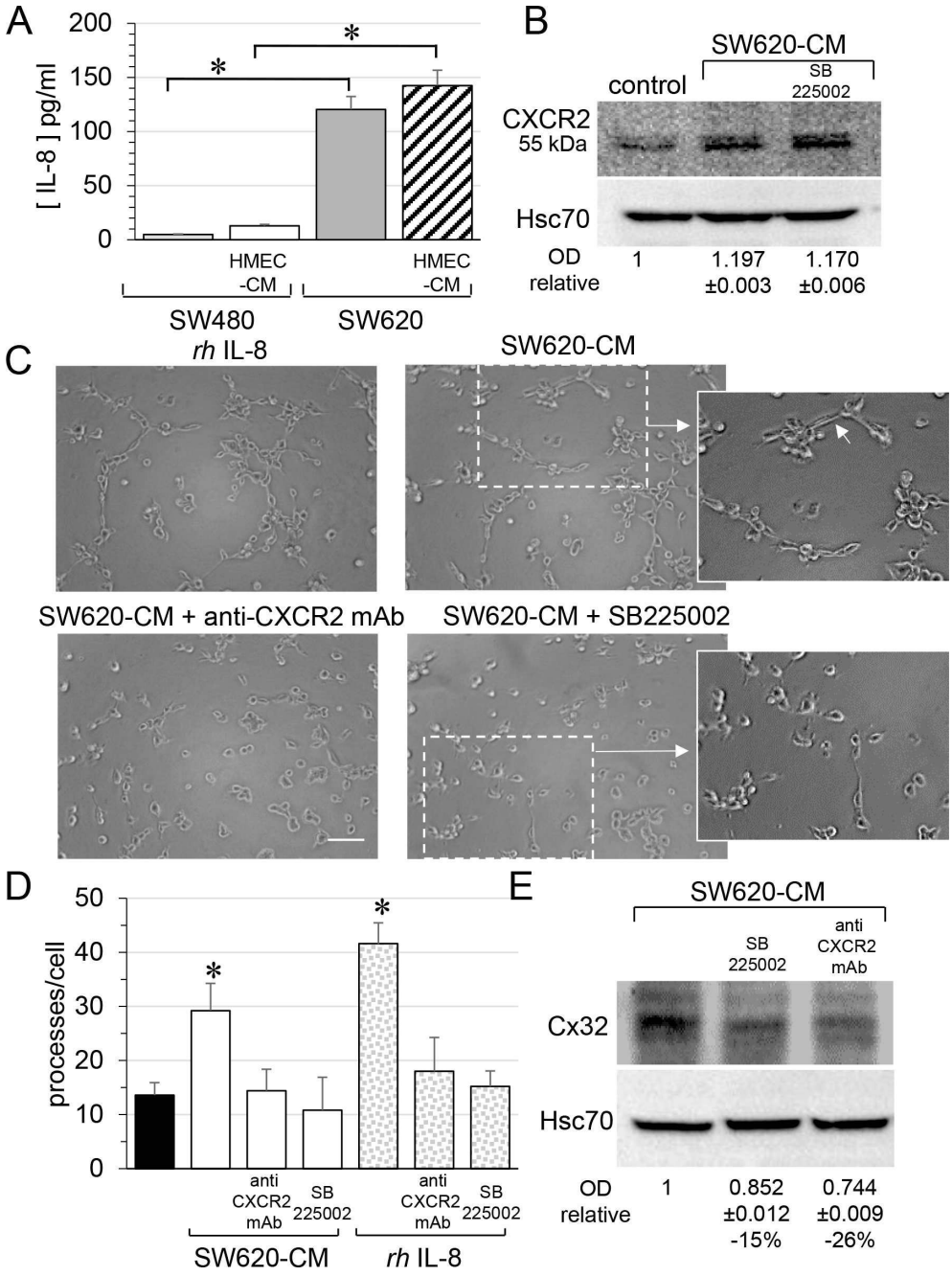

Figure 6: SW620 cell-secreted factors require CXCR2 signaling pathway to induce the endothelial Cx32 expression and tube formation. A. IL-8 secretion in conditioned media from SW480 and SW620 cells was examined through ELISA. CRC cells were exposed or not to the HMEC-CM. All cell media were collected after 6 h (mean ± SD, **P*-values < 0.01 Mann-Whitney U test and Kruskal-Wallis test; $n = 4$). **B.** SW620-CM increase the endothelial expression of the CXCR2 receptor. A slight but significant increase in optical density (OD; relative to control) of bands was detected in response to SW620-CM compared with unstimulated HMEC (*P*-values < 0.01 Mann-Whitney U test and Kruskal-Wallis test; $n = 4$). No inhibitory effect was observed by pre-treating HMEC with SB225002 (200 nM), the CXCR2 antagonist. Representative of 4 experiments (Hsc70 as loading control; 100 µg/lane). **C–D.** Endothelial CXCR2 conveys angiogenic effects of SW620-CM. HMEC were pretreated or not with neutralizing anti-CXCR2 antibody (anti-CXCR2 mAb; 10 µg/ml) or SB225002. Cells were exposed to SW620-CM or human recombinant *rh*IL-8 (1 ng/ml) for 6 h. C. Representative Images of tube formation (Bar 80 µm). The dotted areas are enlarged in the inserts on the right. Arrows indicated branch points. D. Number of branch points per field of view was quantified (mean ± SD, $n = 4$; **P* < 0.01 *vs* control). **E.** Blocking CXCR2 significantly diminished SW620-CM-induced expression of Cx32 in HMEC (*P*-values < 0.01 *vs* SW620-CM Mann-Whitney U test and Kruskal-Wallis test; $n = 3$). HMEC were exposed to cell-conditioned media for 6 h. In some cases, HMEC were pretreated with anti-CXCR2 mAb or SB225002, as indicated. This is a representative of three experiments with similar results (Hsc70 as loading control; 100 µg/lane).

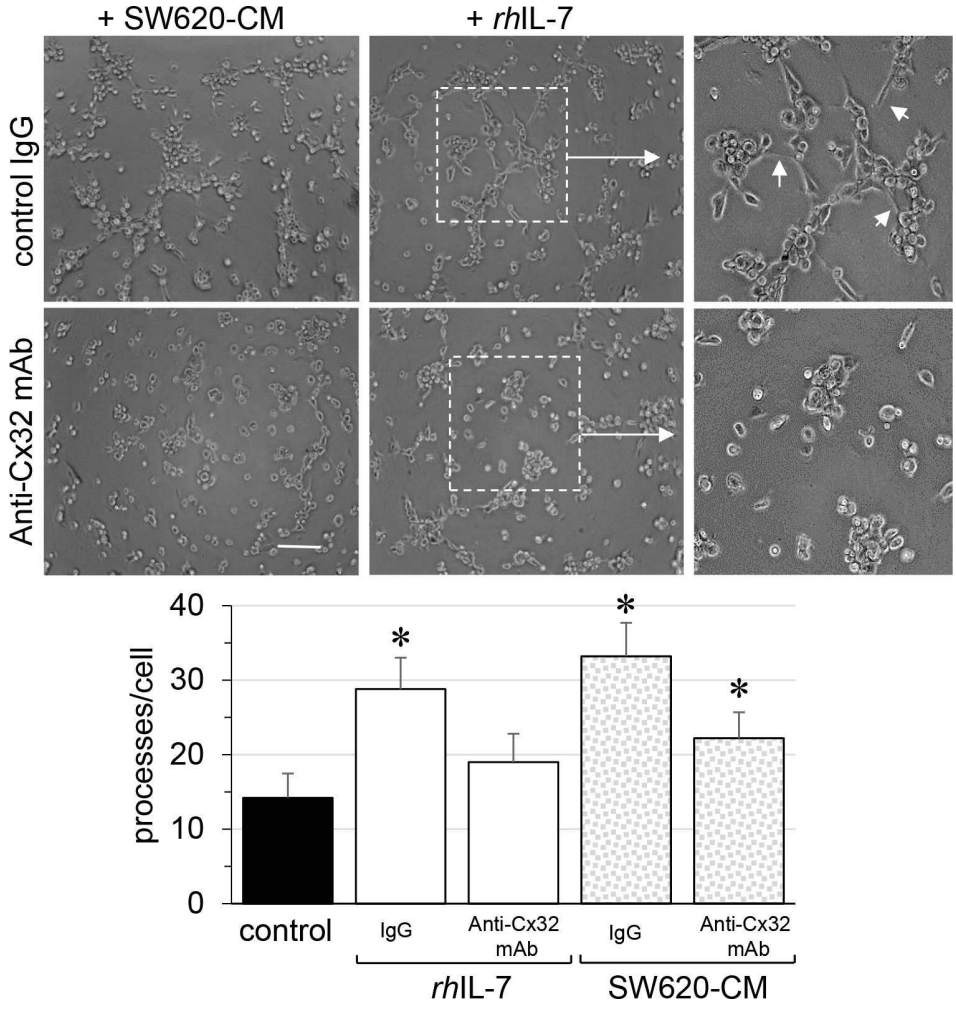

Figure 7: Endothelial Cx32 contributes to angiogenic effects of IL-7 as SW620-CM does. *In vitro* tubulogenesis assay of HMEC pretreated or not (control IgG) with inhibitory monoclonal antibody against Cx32 (0.2 µg/ml anti-Cx32mAb). HMEC were plated on Matrigel-coated 24-well plates, incubated with SW620-CM or human recombinant *rh*IL-7 (1 ng/ml) for 6 h, and photographed (Bar 80 µm). The dotted areas are enlarged in the inserts on the right. Arrows indicated branch points. Histogram shows the number of branch points per field of view (at least 80 single cells were scored; mean ± SD, $n = 4$; $^*P < 0.01$ *vs* control). Blocking Cx32 decreases *rh*IL-7- and SW620-CM-induced tube formation ($^*P < 0.05$ *vs* control; $n = 3$).

DISCUSSION

The gradual loss of functional Cx43 gap junction and the increased expression of Cx32 in colorectal cancer biopsy were previously associated with a worst tumor grading, suggesting a role for these connexins in metastasis formation [11-13]. Here, we demonstrate that tumor cells can affect the expression of Cx proteins in endothelial cells. Cells derived from a primary tumor secrete high levels of HSP27 that promotes the phosphorylation of Cx43 in endothelial cells and the formation of gap junction between tumor and endothelial cells. Cells derived from a metastatic site in the same patient do not modulate Cx43 in endothelial cells but rather promote Cx32 expression and tube formation through a mechanism that involves CXCR2 expression. A model is proposed in Fig. 8.

A unique feature of SW480 and SW620 colon carcinoma cell lines is that they derive from primary and secondary tumors resected from the same patient [15], thus may represent a valuable resource for examining changes late in colon cancer progression [14, 38]. SW480 cells migrate faster than SW620 cells across HMEC monolayers, which is in agreement with their higher locomotion activity [39] and their higher capacity to generate metastasis in a xenograft model [14]. SW480 cells had been shown to release HSP27 whereas SW620 cells did not [16, 17]. HSP27 overexpression has been inversely correlated to metastatic behavior of human colorectal carcinoma (CRC) cells [16, 40-42]. Here, we show that HSP27 released by SW480 cells modulates the phosphorylation of endothelial Cx43, thereby increasing GJIC between SW480 cells and endothelial

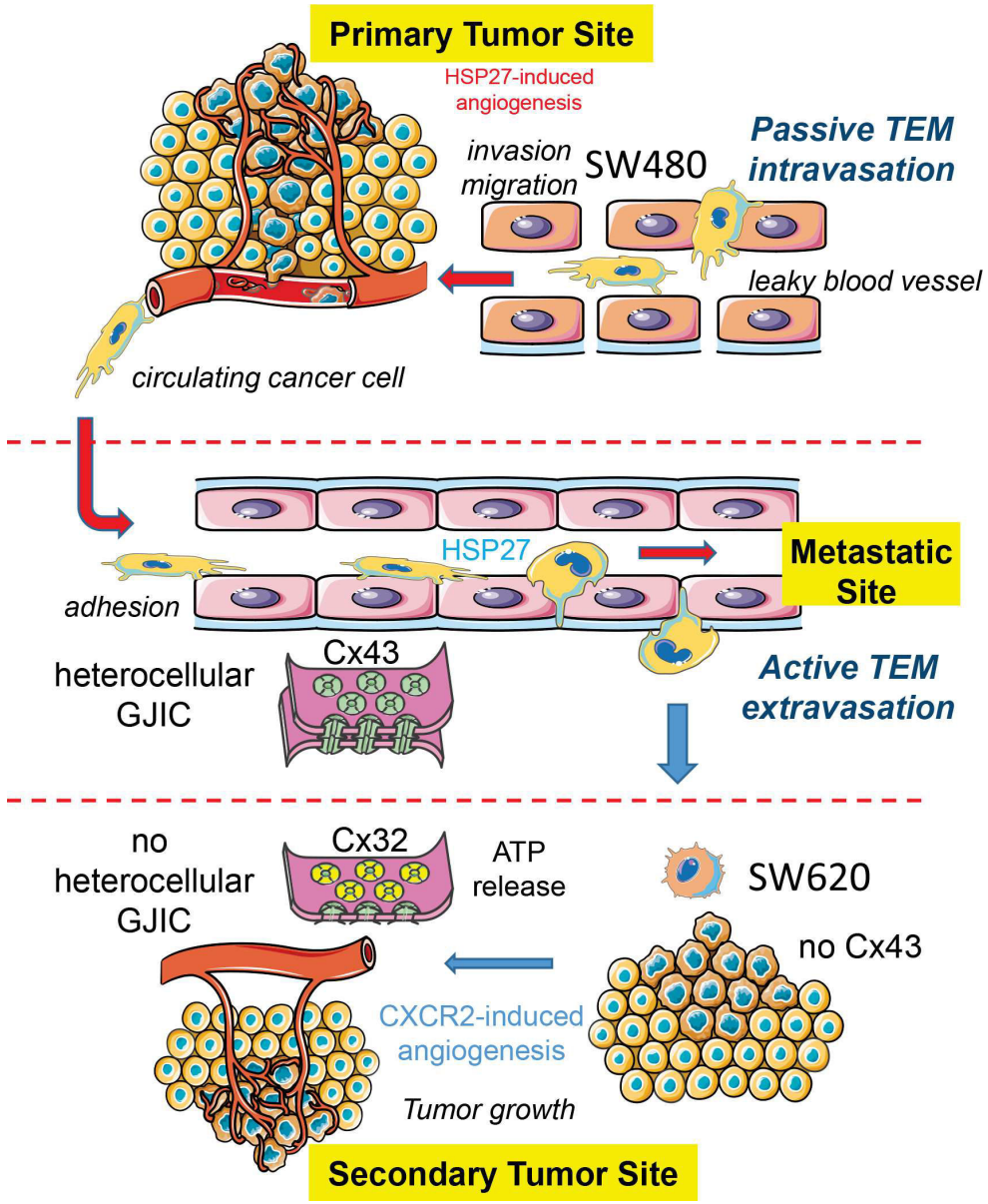

Figure 8: Hypothetical model of the endothelial connexin contribution to the colorectal cancer (CRC) pathogenesis. The diagram shows the endothelial cell (EC) expression of both Cx32 and Cx43 as well as their ability to form hemi-channels or gap junction channels with CRC cells at the microvascular level. Cancer cells from a primary tumor (here, SW480 cells) locally invade the surrounding tissue, enter the microvasculature of the blood system (passive intravasation), survive and translocate through the bloodstream to micro-vessels of distant tissues. SW480 cells release HSP27 that favors the establishment of GJIC, via Cx43-channels, with the underlying endothelium. This direct cell-to-cell communication contributes to their trans-endothelial migration TEM (active extravasation). In contrast, cancer cells from a metastatic site (here, SW620 cells) release larger amount of chemokines, increasing the endothelial expression of the receptor CXCR2. In turn, CXCR2 promotes both endothelial Cx32 expression and tubulogenesis. The release of ATP through Cx32 hemi-channels from ECs and the subsequent ATP-mediated activation of purinergic P2Y2 receptors could modulate crosstalk between ECs and metastatic cancer cells, favoring neo-angiogenesis in metastatic foci.

cells. This heterocellular GJIC may be a necessary step for extravasation of CRC cells from the blood flow to the metastatic site. Several kinases, including protein kinase A, protein kinase C, Cdc2/Cyclin B1 kinase, casein kinase 1, MAP kinase and Src family kinases, were shown to phosphorylate serine residues at the C-terminus of Cx43 [43-45]. While the precise kinase involved in the SW480-CM-induced changes in the phosphorylation of Cx43 serine residues in HMEC was not identified, this event favors 14-3-3 binding to Cx43. Such a binding increases the incorporation of Cx43 multimers into existing gap junctional plaques [23], and facilitates Cx43 channel

formation [24] and GJIC formation between cells. Of note, 14-3-3 protein overexpression also promotes lung cancer progression when combined with HSP27 overexpression [46].

In contrast to HSP27-mediated effects of SW480 cells on Cx43 expression, SW620 cell-secreted factors up-regulate the endothelial expression of Cx32 and enhance tube formation via a CXCR2, suggesting a promoting effect on angiogenesis and, consequently, tumor growth [27]. It remains unknown if a common regulatory mechanism accounts for Cx32 overexpression and Cx43 down-regulation in endothelial cells. A cellular redistribution of Cx32 and Cx43 has been previously associated with the metastasis potential of CRC [11] and breast cancer [9] cells. We show that, by opening Cx32 hemichannels, SW620-CM triggers ATP release, which may not depend on P2X7 receptor activation or pannexin channels that can also release ATP in other cell settings. By activating specific purinergic receptors, ATP released through Cx32 hemichannels could modulate the crosstalk between cancer and endothelial cells, as do P2Y2 receptors in breast cancer metastasis [47].

CXCR2 is another critical component of tumor cell behavior and its expression in endothelial cells favors tumor angiogenesis [48]. In colorectal tumors, CXCR2 was identified on tumor cells, endothelial cells, infiltrating neutrophils, and macrophages [49, 50], and CXCR2 overexpression was identified in CRC liver metastases [51]. CXCR2 promotes tumor growth through recruiting pro-tumorigenic neutrophils and stimulating angiogenesis [52, 53]. A CXCR2 antagonist inhibits proliferation and invasion of CRC cells in an *in vitro* assays and the growth of tumor xenografts in immune-deficient mice [54]. CXCR2 can be activated in an autocrine-dependent manner [55], through one or several of its ligands (i.e. IL-1, 2, and 3, epithelial cell derived neutrophil-activating peptide-78/IL-5, granulocyte chemotactic protein-2/IL-6, IL-7, and IL-8). In addition to IL-8, extensively studied in *in vitro* and *in vivo* CRC cell models [34-36], we show here that IL-7 could be also a potent angiogenic factor that induces tubulogenesis in a Cx32-dependent manner. In breast cancer, IL-7 stimulates invasion and secretion of the lymphangiogenic factors VEGF-C and VEGF-D [56, 57].

To conclude, the differential ability of SW480 and SW620 cells to promote the expression and activation of Cx43 and Cx32, respectively, illustrates the functional heterogeneity of tumor cells in a given patient. Some tumor cells induce the formation of heterocellular GJIC via phosphorylation of Cx43 whereas other promote tubulogenesis via the induction of Cx32 expression. These tumor cells modulate their microenvironment through the release of soluble factors such as soluble HSP27. Further exploration of CRC cell-mediated endothelial junction remodeling may suggest novel approaches for blocking cancer cell migration and metastasis formation.

MATERIALS AND METHODS

Cells

Human microvascular endothelial cells (HMEC; Lonza; Basel, Switzerland) were grown in DMEM plus 10% FCS (5% CO_2; 37°C). Human colorectal cancer cell lines, SW480 (ATCC CCL-228) and SW620 (ATCC CCL-227) were plated in DMEM plus 10% FCS. Untouched cell lines and HMEC were transfected by lipofectamine RNAiMAX (Invitrogen; Life Technologies, Saint-Aubin, Fr). siRNA HSP27 was purchased from Sigma-Aldrich (SASI_Hs01_00051449; Saint-Quentin Fallavier, Fr) and control siRNA was from Dharmacon (Fermentas; ThermoFischer, Saint-Remy-les-Chevreuses, Fr). Cells were incubated overnight in FCS-free media before use.

Reagents

Low endotoxin rhHSP27 was purchased from Enzo Life Sciences (Villeurbanne, Fr) and rabbit anti-HSP27 from ABR (AffinityBioReagent, ThermoFisher, Fr). Recombinant human *rh*IL-8 and *rh*IL-7 were from R&D Systems. Mouse anti-Hsc70 was from Santa Cruz Biotech. Polymyxin B was from InvivoGen (Toulouse, Fr). Rabbit polyclonal anti-Cx43 (710700), mouse monoclonal anti-Cx43 (CX-1B1), anti-Cx32 (CX-2C2) and ZO-1 (ZO1-1A12) antibodies were from Invitrogen. Rabbit oligoclonal anti-Panx-1 (11HCLC) and rabbit polyclonal anti-CIP75 were from ThermoScientific (Rockford, USA) and anti-14-3-3, anti-phosphoserine and anti-CXCR2 from Abcam. DiL-C18, thapsigargin and fura-2/AM were from Molecular Probes. Other chemicals were from Sigma-Aldrich.

Specific cell treatments

To avoid endotoxin contamination of rhHSP27, cells were preincubated with polymyxin B (PMB, 10 µM; 30-60 min) and rhHSP27 solutions were also treated with PMB prior to their use. To block TLR3, HMEC were pre-incubated with the neutralizing anti-hTLR3 (antiTLR3 mAb; 20 µg/ml) for 1 h (eBioscience, San Diego, CA, USA). To block the signaling of TLR2 and TLR4 induced by LPS, OxPAPC (30 µg/ml) was added (InvivoGen). To block CXCR2, HMEC were pretreated with 200 nM SB225002 (CXCR2 antagonist) for 30 min or with 10 µg/ml of neutralizing anti-CXCR-2/IL-8 RD (Clone 48311) for 1 h (R&D Systems). For collection of conditioned media (CM), confluent cells were grown overnight in FCS-free DMEM then fresh medium (4 ml/T-75 flask) was added for 6 h before to be collected.

Fluorescence recovery after photobleaching (FRAP)

The GJIC between HMEC was measured by means of gap-FRAP method [18]. Cells were loaded with 10 ng/µl

of calcein/AM for 15 min. The fluorescence of investigated cells was bleached at 405 nm. The recovery of fluorescence was measured at 488 nm every 20 sec for a time period of 8 min. The fluorescence in one unbleached cell was used to correct the artefact loss of fluorescence. The permeability of gap junctions is estimated by the diffusion rate constant k (expressed in min^{-1}) determined from recovery curves as following: $(Fi - Ft)/(Fi - F0) = e^{-kt}$, where Fi, Ft and F0 are intensities before bleaching, at time t and $t = 0$ respectively.

Transendothelial migration (TEM) assay

HMEC were cultured on 8-µm membrane pores of Transwell inserts in 24-well plates until confluency (CytoSelectTM, Cell Biolabs; Euromedex, Mundolsheim, Fr). CRC cells were seeded on the top of HMEC monolayer (300,000 cells per well). After coculturing for 6 h, invasive cells on the membrane bottom were stained and quantified at OD 560 nm after extraction. Each experiment used triplicate wells and the same assay was repeated four time.

Heterocellular GJIC functionality

CRC cells were labeled with 4 µM calcein/AM (30 min) together with 10 µM DiL-C18 as previously detailed [18]. After washing, 10^3 fluorescent cells were laid on HMEC monolayers. The transfer of dye was visualized after a given time at 37°C.

Immunodetection of protein phosphorylation

Cell were washed 5 times with ice-cold PBS and lysed (45 min on ice) using 1 ml of lysis buffer containing: 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, the phosphatase inhibitor cocktails 2 and 3 (1:100; Sigma-Aldrich) and protease inhibitor mixture (Roche Molecular Biochemical). Cells were scraped, centrifugated, and lysates collected. Proteins were boiled for 5 min in 2 µl SDS buffer, fractionated using 10% SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad, CA). Membranes were blocked for 1 h with 5% BSA in Tris buffered saline with Tween 20 (0.1%) (TBS-T), and incubated overnight with antibodies.

Immunoprecipitation

Briefly, cells were lysed in RIPA buffer, and immunoprecipitation was performed with antibodies, as previously described [19].

Immunofluorescence and imaging

Cells were fixed in 4% PAF and permeabilized with 0.1% Triton X-100 [19]. Images were performed using a Leica SP2 RS confocal microscope (Z-series of 0.6 µm-optical sections; 512x512 pixels; Rueil-Malmaison, Fr). For co-localization, images were taken on Axio Imager 2 (Carl Zeiss GmbH) with an Apotome2 module (Optical sections of 0.5 µm; 512x512; Oberkochen, Germany).

ELISA analyses

HSP27 levels in cell supernatants were evaluated using enzyme-linked immune-absorbent assay (ELISA kit; Enzo Life Sci. ADI-EKS-500) according to the manufacturer's instructions. The quantitative determinations of human IL-8 concentrations were made by enzyme-linked immunosorbent assays (ELISA Quantikine; R&D Systems) as previously described [19].

Endothelial tube formation assay in collagen gels

HMEC were trypsinized and resuspended in ECM gel with DMEM or CRC cell's supernatants according to the manufacturer's instructions (from Cell Biolabs, Inc) [18]. For short term assays after 6 hours of incubation at 37°C, 80 single cells were scored for the number of processes per cell. Each well represents an n of 1 and is duplicated for each experiment, and each experiment was repeated three times. Cells were photographed at a magnification of x10 using Zeiss microscope, equipped with a video camera.

Antibody transfer into HMEC

Inhibitory anti-Cx32 mAb (CX-2C2; Novex by Invitrogen) was transferred into HMEC using the PULSin protein delivery reagent according to the manufacturer's instructions (PolyPlus-transfection, New York, NY). Briefly, HMEC were grown to 70% confluence in 24-well tissue-culture plates and washed with PBS. A mixture of 20 µL of HEPES-buffered saline (HBS) with 0.2 µg anti-Cx32 mAb or control IgG (Sigma) and 0.8 µL PULSin was incubated at room temperature for 15 min. HMEC were incubated with 180 µL Opti-MEM with 20 µL antibody containing solution at 37°C for 4 h before used.

ATP measurement

Concentration of ATP in cell media was detected by luciferin-luciferase assay (ENLITEN ATP Assay, Promega; Charbonnieres, Fr). HMEC were plated at 500 × 103 cells/cm 2, growth arrested in FCS-free medium and exposed to carbenoxolone (200 µM) or GAP26 (500 µM) and/or SW480- or SW620-CM. Supernatants were collected after 6 h, put on ice and centrifuged at 12,000 *g* for 10 min.

Analysis of RNA expression for Panx-1

Total RNA extraction, first-strand DNA synthesis and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) were performed as described previously. The primer sequences are as

follows: FP: CTGTGGACAAGATGGTCACG and RP: CAGCAGGATGTAGGGGAAAA.

Statistical analysis

Results are expressed as mean ± SD. Groups were compared using one-way analysis of variance (ANOVA; Statview Software). Stimulated samples were compared to controls by two-tailed, unpaired *t*-tests. A Mann-Whitney *U* test was also used to compare data groups. In some cases, statistics were made with Tanagra software (http://freestatistics.altervista.org/?e_4) using a Kruskal-Wallis 1-way ANOVA. In all cases, $*P$ values < 0.05 were significant.

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CONFLICTS OF INTEREST

None declared.

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Heat shock proteins as danger signals for cancer detection

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First discovered in 1962, heat shock proteins (HSPs) are highly studied with about 35,500 publications on the subject to date. HSPs are highly conserved, function as molecular chaperones for a large panel of "client" proteins and have strong cytoprotective properties. Induced by many different stress signals, they promote cell survival in adverse conditions. Therefore, their roles have been investigated in several conditions and pathologies where HSPs accumulate, such as in cancer. Among the diverse mammalian HSPs, some members share several features that may qualify them as cancer biomarkers. This review focuses mainly on three inducible HSPs: HSP27, HPS70, and HSP90. Our survey of recent literature highlights some recurring weaknesses in studies of the HSPs, but also identifies findings that indicate that some HSPs have potential as cancer biomarkers for successful clinical applications.

Keywords: heat shock protein, danger signal, detection, biomarker, stress, cancer

INTRODUCTION

Heat shock proteins (HSPs) are important molecular players in the cellular stress response (Macario and Conway de Macario, 2004). They can be divided into five superfamilies according to their molecular weight: small HSPs [or *HSPB* family with the recent ontology (Kampinga et al., 2009)], HSP40 (*DNAJ*), HSP70 (*HSPA*), HSP90 (*HSPC*), and HSP110 (*HSPH*). Two main functions have been described for HSPs: firstly, they act as molecular chaperones thereby playing a role in protein folding, aggregation, transport, and/or stabilization. Secondly, they prevent cell death, for instance by preventing post-mitochondrial apoptosis in caspase-dependent (e.g., HSP27, HSP70, and HSP90) and/or independent (e.g., HSP70) pathways (Gallucci and Matzinger, 2001). HSPs were first discovered in *Drosophila melanogaster* as a set of proteins whose expression was induced by heat shock. They are highly conserved proteins, present in the three domains of life: archaea, bacteria, and eukaryotes (Macario and Conway de Macario, 2004). HSPs are now also called stress proteins since their expression was found to be induced in response to a wide variety of physiological and environmental insults allowing cells to survive to otherwise lethal conditions. Stressors can be physical and chemical insults such as radiation, including ultraviolet light and magnetic fields, compression, shearing and stretching, hypoxia, pH shift, nutrient deprivation, or exposure to reactive oxygen species, alcohols, or metals. They also include biological insults such as fever, cold, infection, inflammation, diseases including cancer, cardiac diseases, and neurodegenerative disorders. Cellular stress can also be triggered by treatments with anticancer drugs or antibiotics (Ciocca and Calderwood, 2005; Macario and Conway de Macario, 2005; Garrido et al., 2006; Jego et al., 2010; Willis and Patterson, 2010; Macario et al., 2011). HSPs are reported to be overexpressed in several pathologies. As such, members of the large HSP family are studied widely as they may represent interesting biomarker candidates. Chaperones such as HSP70 and HSP90 tend to be team-players, acting together with other chaperones and cochaperones (Macario and Conway de Macario, 2005). Chaperone–cochaperone complexes such as HSP70, HSP40, and nucleotide-exchange factor are able to help nascent polypeptide chains to fold properly, refold damaged molecules, and can also direct proteins to a protein-degrading mechanism such as the ubiquitin–proteasome system (Lanneau et al., 2010). Each HSP is localized in one or a set of specific compartment(s). For instance, HSP70 and HSP90 can be present in the cytosol and the nucleus, whereas HSP60 is found in mitochondria (see **Figure 1**) and grp78 (Bip, or *HSPA*5) in the endoplasmic reticulum. Microorganisms may be another source of detectable HSPs. Indeed, humans may be considered as a "superorganism," colonized by around a thousand different species of microorganisms. These are mostly bacterial cells present in the intestinal tract. These bacterial cells outnumber our cells by at least a factor of 10, (the total number of cells comprising the adult human body, both native and foreign, being estimated to $\sim 10^{14}$) and their genes outnumber our own by a factor of 100. These HSPs from microorganisms often induce a proinflammatory response. Thus, when measuring anti-HSP levels, especially against a conserved constitutive chaperone such as HSP60, it is important to consider the protein may originate from microorganisms. In this review we will focus on HSP60 and the three most inducible HSPs: HSP27, HSP70, and HSP90.

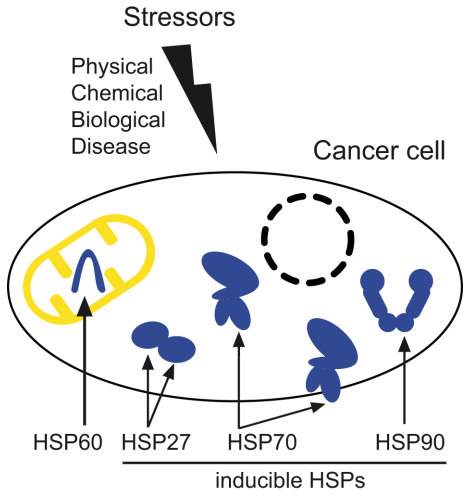

FIGURE 1 | Stressors of physical, chemical, or biological origin, as well as diseases such as cancer can induce the accumulation of heat shock proteins necessary for the survival of cancer cells. This scheme illustrates the main localizations of HSP60, present in the mitochondria, and the three inducible cytosolic/nuclear HSPs: HSP27, HSP70 (also found in the cell membrane), and HSP90.

EXTRACELLULAR HSPs: CIRCULATING AND MEMBRANE-BOUND HSPs

Due to their lack of a transmembrane domain, HSPs are considered intracellular soluble proteins (Gallucci and Matzinger, 2001) that can be induced by a wide panel of stress signals and have strong protective properties. However, HSPs such as HSP27, HSP60, HSC70, HSP70, and HSP90 can also be found in the extracellular environment where their role is believed to be immunogenic (Schmitt et al., 2007). How HSPs are found in the extracellular medium is still a debated issue. HSPs lack the consensus signal for secretion via the classical Golgi pathway. The first hypothesis was attributed to spontaneous cell death leading to HSP extravasation (Basu et al., 2000). However, it was found that undamaged, live cells released HSP70 by an active non-classical secretory pathway that was not affected by ER Golgi system inhibitors such as brefeldin A (Hightower and Guidon, 1989; Hunter-Lavin et al., 2004). Thus, active release of HSP70 might occur through one or more alternative mechanisms (Nickel and Seedorf, 2008). Different pathways have also been proposed for HSP70 release: a lysosome–endosome pathway (Mambula and Calderwood, 2006) or a release by secretory-like granules (Evdonin et al., 2006), but the bulk of the evidence points to an insertion of HSP70 oligomers into the lipid bilayer of export vesicles. These oligomers form ATP-dependent ion channels, leaving only a small C-terminal region of the protein outside (Vega et al., 2008). Thus, several mechanisms have been proposed to account for the release of these HSPs into biofluids, however the question remains unresolved (Didelot et al., 2007; Joly et al., 2010; De Maio, 2011).

Some HSPs can also be found on the cell surface, such as HSP70. This has been widely reported in a variety of conditions, especially for cancer and immune cells (De Maio, 2011). For cell surface display to occur, a high intracellular HSP level is thought to be necessary. For display of HSP70, for example, only about 10% of the intracellular HSP70 is translocated to the cell membrane. The high specificity of HSP70 and HSC70 for membranes seems to be related to membrane fluidity and thus to lipid composition of the bilayer, due to affinity for phosphatidylserine and cholesterol (Arispe et al., 2004; Schilling et al., 2009). Presence of HSP70 in the membranes was stable even when treated with non-ionic detergent, suggesting its insertion into lipid rafts (Hunter-Lavin et al., 2004; Vega et al., 2008). In contrast to intracellular HSPs that mainly play a cytoprotective role, some extracellular HSPs such as HSP27 and HSP70, have immunogenic properties (Didelot et al., 2007) and may induce either a pro- or an anti-inflammatory response. Because cell membranes can contain a certain amount of HSPs, exosomes, and other vesicles derived from these membranes also harbor HSPs on their membranes. For example, membrane-bound HSP70 in vesicles were shown to activate macrophages (Vega et al., 2008). We have recently shown that tumor-derived exosomes harboring HSP70 in the membrane triggered STAT3 activation in myeloid-derived suppressive cells, leading to tolerance of the immune system to the tumor cells (Chalmin et al., 2010). Such tumor-derived exosomes displaying HSPs may provide interesting options for cancer detection and need to be further explored.

BIOMARKERS

A useful biomarker needs to fulfill several criteria (Seigneuric et al., 2010). It should ideally be at least: (i) overexpressed and positively associated with the pathology of interest in order to identify cases, (ii) specific to the pathology of interest, (iii) relatively easy to measure, ideally by non-invasive assays, and (iv) induced as early as possible to enable early detection.

The most widely used techniques for the investigation of extracellular HSPs as cancer biomarkers have been: immunohistochemical (IHC) stainings to detect the presence of a given HSP from a biopsy, tissue microarrays (TMA), western blot (WB) analysis, and enzyme-linked immunosorbent assay (ELISA), which allows HSP levels to be quantified down to a few nanograms per milliliter, but requires labels that may denature the protein of interest.

HSP27

HSP27 belongs to the small heat shock protein family. Its structure and function are thought to be modulated by phosphorylation mediated by MAPK2. Intracellular HSP27 plays an anti-apoptotic role through interaction with Bid or cytochrome c (Bruey et al., 2000) and also has a main role as a chaperone, preventing the aggregation of misfolded proteins. As such, HSP27 may contribute to the pathogenesis of human diseases such as cancer, autoimmune diseases, neurological disorders (e.g., Alzheimer's disease), and cardiovascular diseases, where HSP27 has been investigated as a biomarker for myocardial ischemia (Ghayour-Mobarhan et al., 2011). In the context of oncology, HSP27 has been detected in both the intracellular and extracellular environment. An increased level of HSP27 has been reported in ovarian cancer, and more frequently in prostate and breast cancers. Overexpression of HSP27 was found to correlate with poor prognosis for patients with these diseases (Langdon et al., 1995; Calderwood, 2010; Khalil et al., 2011). HSP27 was also reported to contribute to invasion and metastasis (Xu et al., 2006), suggesting that HSP27 could be a biomarker for the diagnosis of cancer (Lee et al., 2005).

In a study of biopsy samples from prostate cancer patients, expression of intracellular HSP27 was reported using IHC stainings (see **Table 1**). It was observed that HSP27 expression in prostate cancer tissue was up-regulated compared to the controls (Miyake et al., 2006). Similar qualitative results were also obtained in breast cancer (Rui et al., 2003). HSP27 levels in serum and/or tumor microenvironment were determined by ELISA. Serum levels of HSP27 were significantly higher ($P < 0.001$) in breast cancer patients ($1{,}038.38 \pm 155.37$ pg/mL; $n = 32$) compared to healthy controls (256.29 ± 54.01 pg/mL; $n = 26$). Interestingly, HSP27 released by patients' tumor cells were significantly greater ($P < 0.0001$) than that measured in the serum with $24{,}220 \pm 4{,}796$ pg/10^6 cells/mL ($n = 7$) and $1{,}459 \pm 471$ pg/10^6 cells/mL ($n = 7$), respectively. Another study of breast cancer patients showed that HSP27 levels in the interstitial fluid isolated from primary breast tumor could be extremely high: $2{,}615{,}428 \pm 566{,}442$ pg/mL; ($n = 7$). This concentration was more than: 2,500-fold higher than that detected in patients' serum, more than 100-fold that of patients' breast tumor culture supernatants, and more than 25-fold higher than the HSP27 level detected in the normal breast tissue interstitial fluid ($103{,}600 \pm 35{,}702$ pg/mL). Although the number of patients was very small ($n = 3$), this preliminary study suggests highly elevated levels of soluble HSP27 in the human primary breast tumor microenvironment (Banerjee et al., 2011). It would be interesting to confirm this finding with a larger cohort of patients.

HSP60

HSP60, initially called chaperonin, was one of the first chaperones studied. It plays an essential role in the transport and folding of mitochondrial proteins, and is reported to be associated with different cancers (see **Table 2**).

Clinical data from patients with localized and locally advanced prostate cancer showed an association between IHC expression of HSP60 and tumor progression. HSP60 expression was also reported to be highly associated with androgen independence in the group of locally advanced cancers with androgen ablation (Castilla et al., 2010). The intensity and extent of immunoreactivity of HSP60, estimated by TMA analyses, significantly predicted biochemical recurrence. It was shown that, for patients with intense HSP60 staining in biopsy, recurrence-free survival was shorter than in those with weak expression (Glaessgen et al., 2008). A study performed in human prostate cancers indicates that HSP60 expression assessed via IHC increases in both early and advanced prostate cancer when compared with non-neoplastic prostatic epithelium (Cornford et al., 2000). The semi-quantitative evaluation of HSPs expression level showed no association with Gleason score neither for the early nor for the advanced cancers. Data from WB analyses of whole lysates in prostate cancer cell lines was consistent with data from tissue specimens. Indeed, each of the malignant cell lines tested showed an increased HSP60 expression but no identifiable difference in relative expression between stage or grade of the individual cancers.

The prognostic significance of HSP60 in cervical cancer caused was assessed by 2-DE, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and WB analyses (Hwang et al., 2009). The results from 2-DE proteomics, confirmed by WB analyses ($P < 0.05$), suggest that HSP60 may be involved in the development of cervical cancer.

Autoantibodies may be an excellent tool for the early diagnosis of cancer. Many studies have investigated the tumor-associated autoimmune response to identify new early diagnostic markers. In breast cancer patients, ELISA experiments showed the presence

Table 1 | Selection of recent publications assessing HSP27 as a biomarker in cancers.

Sample type	Cancer type	Total number of samples	Assay	Finding/claim (References)
Biopsy	Prostate	97 patients	IHC	HSP27 expression level was significantly associated with Gleason score, but not with studied factors before radical prostatectomy (Miyake et al., 2006)
Tissue	Prostate	120 patients; 60 controls	IHC	The level of HSP27 expression was correlated with their Gleason grade and associated with poor clinical outcome (Cornford et al., 2000)
Tissue	Bladder	42 patients; 10 controls	IHC	No correlation between HSP27 expression and grade was found (Lebret et al., 2003)
Tissue	HCC	38 patients	IHC, DI	HSP27 expression increased with the progression of hepatitis B virus-related HCC (Lim et al., 2005)
Tissue	Prostate	193 patients	IHC	HSP27 expression significantly associated with several conventional prognostic factors (Miyake et al., 2010)
Serum	Breast	32 patients; 26 controls	ELISA	Higher levels of HSP27 released in the tumor microenvironment compared to serum levels of cancer patients (Banerjee et al., 2011)
Serum	Breast	76 patients; 54 controls	2-DE; MALDI–TOF–MS	HSP27 was up-regulated in the serum of breast cancer patients (Rui et al., 2003)
Serum	Ovarian	158 patients; 80 controls	ELISA	The mean concentration of anti-HSP27 antibodies was significantly higher than in the control group (Olejek et al., 2009)

Techniques are: 2-DE, two-dimensional gel electrophoresis; DI, dot immunoblot; ELISA, enzyme-linked immunosorbent assay, IHC, immunohistochemical analyses; MALDI–TOF–MS, matrix-assisted laser desorption/ionization – time-of-flight – mass spectrometry. HCC stands for hepatocellular carcinoma.

of autoantibodies against HSP60 and the level of serum HSP60 antibodies was found to be dependent on the cancer grade. Interestingly, high grade tumors showed a more elevated level of HSP60 autoantibodies compared to low-grade tumors. HSP60 mRNA levels were significantly higher in primary breast cancer compared to healthy breast tissues. Using IHC, it was found that HSP60 expression increased from normal to invasive tissues (Desmetz et al., 2008). Based on a serological proteomics-based approach, a humoral immune response in patients with breast cancer related to HSP60 was found (Hamrita et al., 2008). These authors looked for the presence of IgG antibodies against MCF-7 cell line proteins. They found a higher level for the molecular chaperone HSP60. In line with these results, IHC analyses performed on breast cancer biopsies showed increased expression of HSP60 in tumors of advanced clinical stage when compared with earlier stage carcinomas (Isidoro et al., 2005). These results support the fact that HSP60 overexpression during the initial stages of breast carcinogenesis may be clinically relevant for the early diagnosis of breast cancer. HSP60 was also identified by antibodies in sera from patients with chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma. However, HSP60 antibodies in precancer conditions were not a useful candidate as a biomarker for pancreatic cancer. Further investigations should be performed to determine the quantitative changes of this chaperone protein associated with early events leading to tumorigenesis (Looi et al., 2008). In colorectal carcinoma, the elevated expression of HSP60 showed a significant association with tumor differentiation, and may indicate a worse prognosis (Mori et al., 2005).

The correlation of HSP60 expression with tumor growth and/or progression makes this chaperone protein a potential biomarker. However, more efforts should be devoted to quantitative analyses of HSP60 expression to resolve existing contradictory findings concerning its association with a good or a poor prognosis (see **Table 2**).

HSP70

The HSP70 superfamily consists of at least 13 members (Kampinga et al., 2009). Inducible HSP70 (*HSPA1A* or HSP72), an extensively studied, powerful ATP-dependent chaperone with key anti-apoptotic properties (Garrido et al., 2006; Didelot et al., 2007;

Table 2 | Selection of recent publications assessing HSP60 as a biomarker in cancers.

Sample type	Cancer type	Total number of samples	Assay	Finding/claim (References)
Biopsy	Prostate	107 patients	WB; IHC	HSP60 is overexpressed in tumors and strongly associated with prognostic clinical parameters (Castilla et al., 2010)
Tissue	Cervical	20 patients; 20 controls	2-DE; RT-PCR; WB	Increased HSP60 expression (Hwang et al., 2009)
Tissue	Prostate	289 patients	IHC; TMA	HSP60 overexpression was correlated with both biochemical recurrence and Gleason score (Glaessgen et al., 2008)
Tissue	Bladder	42 patients; 10 controls	IHC	HSP60 low expression levels correlated with higher tumor stage. Loss of HSP60 expression was correlated with tumor infiltration (Lebret et al., 2003)
Tissue	Breast	149 patients	IHC; TMA	No association was found between HSP60 and prognosis (Sebastiani et al., 2006)
Tissue	Colorectal	44 patients	cDNA microarray; IHC	A significant association of HSP60 with tumor differentiation and pT stage was observed (Mori et al., 2005)
Tissue, cell line	Prostate	120 patients; 60 controls	IHC; WB	No correlation was found between levels of HSP60 expression and phenotypic behavior of individual primary prostatic cancers (Cornford et al., 2000)
Tissue	Breast	101 patients; 13 controls	WB; IHC	Increased expression of HSP60 compared with controls. HSP60 correlated with patient overall survival (Isidoro et al., 2005)
Tissue	HCC	38 patients	IHC, DI	Expression of HSP60 decreased during hepatocarcinogenesis (Lim et al., 2005)
Serum, tissue, cell line	Breast; ovarian; prostate; ductal carcinoma	147 patients; 93 controls	ELISA; 2-DE; WB; RT-PCR; IHC	HSP60 was overexpressed during the first steps of breast carcinogenesis (Desmetz et al., 2008)
Serum cell line	Breast	40 patients; 42 controls	IHC; MALDI–TOF–MS; 2-DE	Significantly higher level of autoantibodies against HSP60 in breast cancer patient sera (Hamrita et al., 2008)

Techniques are: 2-DE, two-dimensional gel electrophoresis; DI, dot immunoblot; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemical analyses; MALDI–TOF–MS, matrix-assisted laser desorption/ionization – time-of-flight – mass spectrometry; RT-PCR, reverse transcription polymerase chain reaction; TMA, tissue microarray; WB, western blot.

Seigneuric et al., 2011). HSP70 could be an interesting biomarker because its overexpression in serum is associated with many cancers. However, these studies are mostly qualitative. HSP70 is considered to be the most universally stress inducible HSP (Suzuki et al., 2006) with reported inductions of over 200-fold (Modi et al., 2007). A few clinical articles provide quantitative data on HSP70 expression based on ELISA tests (see **Table 3**). The relatively large case–control study nested in the Japan Collaborative Cohort Study for Evaluation of Cancer Risk (Suzuki et al., 2006) reports HSP70 levels in the serum of lung cancer patients in comparison with the C-reactive protein (CRP). The data indicate mean levels of 2.41 ng/mL ($n = 189$) for cases versus 2.01 ng/mL ($n = 377$) for controls. This is to be compared to smaller differences in the CRP levels: 0.92 ng/mL ($n = 209$) versus 0.81 ng/mL ($n = 425$) respectively (Suzuki et al., 2006). In colorectal cancer, the difference in serum levels of HSP70 between patients who survived (1.51 ng/mL, $n = 95$) compared to patients who did not survive (1.84 ng/mL, $n = 84$) was reported to be significant ($P = 0.014$; Kocsis et al., 2010). ELISA-determined concentrations of serum HSP70 autoantibody were reported to be significantly higher in esophageal squamous cell carcinoma (ESCC) patients (0.412 mg/mL, $n = 16$) than for patients with gastric cancer (0.236 mg/mL, $n = 17$, $P < 0.001$), colon cancer (0.231 mg/mL, $n = 19$, $P < 0.001$), or healthy individuals (0.207 mg/mL, $n = 13$, $P < 0.001$; Fujita et al., 2008). Albeit limited in the number of patients and controls, this study suggests the presence of autoantibody against HSP70 in the serum of ESCC patients.

HSP90

HSP90 is an ATP-dependent chaperone that has been highly studied for anticancer therapy (Kamal et al., 2003; Solit and Chiosis, 2008; Jego et al., 2010) with targeted inhibitors being tested in clinical trials in phase II/III. HSP90 ensures the quality control of many proteins involved in cell-signaling pathways. It is also essential for the stability and function of many oncogenic client proteins (Taipale et al., 2010). Its expression in malignant cells is reported to be high and constitutive, suggesting a crucial role in survival and growth of cancer cells (Whitesell and Lindquist, 2005). Studies have investigated the potential prognostic value of HSP90 in different cancers as shown in **Table 4**. In invasive breast cancers, immunohistochemical analyses showed that HSP90 was abundantly expressed in all tumor samples included in the study (Song et al., 2010). A trend toward correlation ($P = 0.062$) was identified between HSP90 expression and diminished relapse-free survival (RFS) in the triple negative subtype (HER-, ER-, PR-). In patients with HER+ (tumors HER2-clustered), the coexpression of HSP90 and PI3K-p110 (or loss of PTEN) predicted a significantly decreased RFS. In line with these results, it was suggested that HSP90 overexpression in malignant breast cancer was associated with decreased survival (Pick et al., 2007). Concerning hepatocellular carcinoma, Lim et al. (2005) suggested that HSP90 expression increases along with the progression of hepatocarcinogenesis. In fact, HSP90 expression showed a strong correlation with prognostic factors of hepatocellular carcinoma, being associated with vascular invasion and metastasis. Similar findings were observed in bladder cancer, where HSP90 level was correlated with

Table 3 | Selection of recent publications assessing HSP70 as a biomarker in cancers.

Sample type	Cancer type	Total number of samples	Assay	Finding/claim (References)
Biopsy	HCC	176 patients	IHC	A panel of HSP70 + glypican 3 + glutamine synthetase proved useful to detect well-differentiated HCC in biopsy (Di Tommaso et al., 2009)
Tissue	Bladder	42 patients; 10 controls	IHC	No correlation was found with tumor grade, disease stage, and patient outcome (Lebret et al., 2003)
Tissue	Prostate	193 patients	IHC	Significant association with either no or limited prognostic parameters (Miyake et al., 2010)
Tissue	Prostate	120 patients; 60 controls	IHC	No correlation was found between levels of HSP70 expression and phenotypic behavior of individual primary prostatic cancers (Cornford et al., 2000)
Tissue	HCC	38 patients	IHC, DI	Positive correlation between HSP70 expression and prognostic factors of hepatitis B virus-related HCC (Lim et al., 2005)
Serum	Lung	189 patients; 377 controls	ELISA	High levels of serum HSP70 might be associated with increased risk of lung cancer among Japanese males (Suzuki et al., 2006)
Serum	ESCC	16 patients; 13 controls	ELISA; 2-DE; WB; MALDI–TOF–MS, IHC	Concentrations of serum HSP70 autoantibody were significantly higher for patients with ESCC than for patients with gastric or colon cancer or healthy individuals (Fujita et al., 2008)
Serum	Colorectal	179 patients	ELISA	Serum level of soluble HSP70 may be a stage-independent prognostic marker in colorectal cancer without distant metastasis (Kocsis et al., 2010)

Techniques are: 2-DE, two-dimensional gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemical analyses; MALDI–TOF–MS, matrix-assisted laser desorption/ionization – time-of-flight – mass spectrometry; DI, dot immunoblot; WB, western blot. ESCC, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma.

high grade tumor (Lebret et al., 2003). In studies on ovarian cancer, HSP90 expression was associated with higher stages, and so may be a good indicator of aggressiveness when associated with other HSPs (Elpek et al., 2003).

HSPs AS BIOMARKERS: HEADING FOR GOLD (STANDARDS)?

The ever increasing body of data dealing with HSPs reflects their potential as both therapeutic targets and as biomarkers. We have highlighted only some of the many interesting points, and identified some pitfalls that are recurrent in many research fields (Ioannidis, 2005; Mischak et al., 2010), and which should be addressed to clarify future work concerning HSP detection.

For the validation of most results presented here, it is crucial that large-scale studies be performed to confirm these findings (Ioannidis, 2005; Mischak et al., 2010). This review of recent research articles on HSP detection in cancer reveals that the most frequently used techniques are essentially qualitative. Overall, IHC staining seems the most frequently used, regardless of the HSP of interest. The two main disadvantages of IHC staining are that the results are qualitative and subjective, like any image analysis. ELISA tests should be favored to provide more objective, quantitative data.

How specific are HSPs as biomarkers in cancer? HSPs are key proteins that tend to be overexpressed in response to a large panel of stressors. They are thus by definition not specific to cancer. However, it is shown that HSPs are necessary for cancer cell survival, making cancer cells in fact addicted to these cytoprotective chaperones. As a consequence, cancer cells express high levels of HSPs compared to normal cells, and need them for survival. It is clear that no single biomarker will be sufficient to detect cancer, its relapse or monitor a treatment (Seigneuric et al., 2010). An alternative, then, is to combine different biomarkers on the same level, (e.g., at the protein level) as exemplified by DNA microarrays and "omics" in general (Seigneuric et al., 2009). Another way to deal with the inherent lack of specificity of a single protein is to combine it with biomarkers from

Table 4 | Selection of recent publications assessing HSP90 as a biomarker in cancers.

Sample type	Cancer type	Total number of samples	Assay	Finding/claim (References)
Tissue	Bladder	42 patients; 10 controls	IHC	HSP90 expression levels correlated with tumor infiltration (Lebret et al., 2003)
Tissue	HCC	38 patients	IHC; DI	Positive correlation between HSP90 expression and prognostic factors of hepatitis B virus-related HCC (Lim et al., 2005)
Tissue	Breast	212 patients	IHC	Expression of HSP90 was associated with 5-year relapse-free survival (RFS). Coexpression of HSP90 and PI3K or expression of HSP90 along with PTEN loss demonstrated prognostic significance in terms of RFS in patients with HER2-positive cancers, but not with HER2-negative cancer (Song et al., 2010)
Tissue	RCC	153 patients	IHC	HSP90 was expressed in most RCC specimens. No significant association with conventional diagnostic factors (Sakai et al., 2009)
Tissue	Prostate	193 patients	IHC	No significant association of HSP90 expression levels with conventional diagnostic factors (Miyake et al., 2010)
Tissue	Ovarian	52 patients	IHC	HSP90 expression was associated with higher stages but did not correlate with prognosis (Elpek et al., 2003)
Tissue cell line	Breast	655 patients	IHC; WB	High HSP90 expression was associated with decreased survival in primary breast cancer (Pick et al., 2007)

Techniques are: IHC, immunohistochemical analyses; DI, dot immunoblot; WB, western blot. HCC, hepatocellular carcinoma; RCC, renal cell carcinoma.

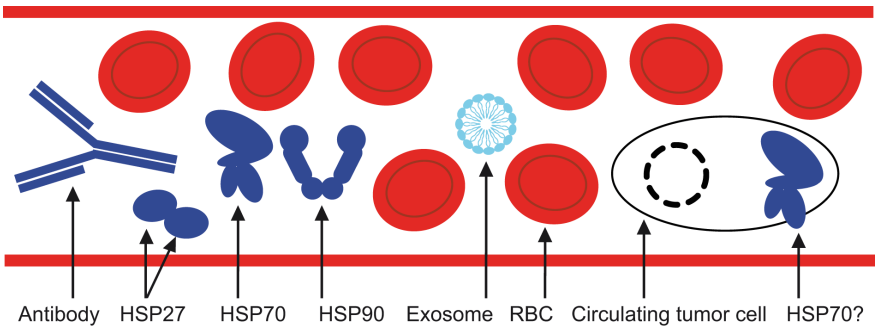

FIGURE 2 | Peripheral blood is a biological fluid of high interest for HSP detection in oncology since its composition besides red blood cells or RBCs is reported to include, circulating HSP antibodies and autoantibodies, HSP27, HSP70, and HSP90, as well as tumor-derived exosomes expressing HSP70 at the membrane. The presence of circulating tumor cells expressing HSP70 at their membrane remains to be demonstrated but may be complementary to the EpCAM biomarker, which is currently in clinical trials.

other levels (see **Figure 2**). For instance, HSP expression at the protein level could be integrated with the measurement of tumor-derived exosomes expressing HSP70 in the membrane. Circulating tumor cells (CTCs) represent another very interesting level. Critical to embryogenesis, cancer cells are thought to undergo an epithelial-to-mesenchymal transition (EMT) during carcinogenesis, contributing to invasion and metastasis (Thiery, 2002; Thiery et al., 2009). Indeed, altered cell–cell and cell–extracellular interactions would enable: (i) the release of epithelial cells, or the "seed" (Fidler, 2003) from the surrounding tissue, (e.g., primary tumor), (ii) entering the circulation (intravasation), (iii) exiting the circulation (extravasation), (iv) the colonization of distant organs (Fidler, 2003). Current clinical investigations into the detection of CTCs are based on the cell receptor EpCAM (Seigneuric et al., 2010). However, EpCAM expression is supposed to decrease during the circulation of CTCs in peripheral blood (or lymph), as reported with flow cytometry experiments (Rao et al., 2005). This may lead to suboptimal detection of CTCs. The process of EMT is well documented in cell lines and mice experiments, but its clinical relevance remains debated (Ledford, 2011). In contrast to normal cells, epithelial cancer cells circulating in a liquid environment should have increased levels of membrane HSP. Although this hypothesis remains to be tested, it would represent an interesting means to robustly detect CTCs based on HSPs expression at the cell membrane. HSP70 is a strong candidate as various cancer cell lines express a fragment of HSP70 at their membrane that can be detected by an antibody (Stangl et al., 2011). This is of high interest for cancer detection, as it enables a means of characterization by flow cytometry and ELISA tests.

Heat shock proteins are essential proteins that are highly conserved, but their expression levels between individuals is reported to differ widely for HSP60 (Shamaei-Tousi et al., 2007), HSP70 (Modi et al., 2007), and/or HSP90 (Ramanathan et al., 2010). The problem of this intrinsic variability in HSP expression may be circumvented by using time series, assessing HSPs with longitudinal data by following-up every single patient as a function of time. In order to compare HSP levels between patients, adjusting for age, gender, and ethnicity may be necessary. The role of circulating HSP70 and/or HSP70 antibodies in the peripheral circulation in the context of aging is interesting (Pockley et al., 1998). Reported ranges of HSP70 expression largely differ. Indeed, discrepancies up to a few orders of magnitude, varying from a few ng/mL (Terry et al., 2006) to values 10 to a 100 times higher were reported (Rea et al., 2001; Njemini et al., 2005), possibly due to ELISA matrix defects (Njemini et al., 2005). However, the serum concentration of HSP70 is reported to decrease with age (Rea et al., 2001; Macario and Conway de Macario, 2005; Terry et al., 2006; Njemini et al., 2011) as is serum HSP60 (Macario and Conway de Macario, 2005). The gender issue should be also addressed. For instance, a 3.5 times higher expression of circulating HSP60 was found in women's peripheral blood compared to men (Macario and Conway de Macario, 2005). In addition, HSP70 serum concentrations in women were about twice those in men (Pockley et al., 1998). The study of Suzuki et al. (2006) reported differences in serum HSP70 levels in cancer patients. The authors reported 2.46 ng/mL ($n = 146$) in male versus 2.25 ng/mL ($n = 43$) in females. For healthy controls however, serum HSP70 levels were quite similar: 2.00 ng/mL ($n = 285$) and 2.06 ng/mL ($n = 92$) respectively (Suzuki et al., 2006).

For an HSP to qualify as a cancer biomarker, its expression level must be compared to gold standards or to commonly assessed biomolecules. A circulating HSP should be compared to other proteins such as CRP, a systemic and non-specific marker of chronic inflammation (Chaturvedi et al., 2010) used daily in practice, or CA 15-3 (cancer antigen 15-3) or CA125 (cancer antigen 125), which are widely used biomarkers for breast (Duffy, 2006) and ovarian cancer (Petricoin et al., 2002), respectively.

CONCLUDING REMARKS

Research studies in cancer detection rely heavily on the identification of proteins, but how many proteins are in fact uniquely expressed by cancer but not normal cells? Presumably, their proteomes are shared at least to some extent, thus favoring quantitative rather than qualitative techniques to reveal subtle differences in expression levels currently found in the pico- to sub-nanomolar range (Krueger, 2006). It is likely that HSPs will not discern different cancer types. However, this may be seen as the major advantage of HSPs: a potentially universal family of cancer biomarkers.

Since the data amassed during the past half century is essentially qualitative, it is too early to suggest which HSP or combination of HSPs may show promise in the future. Yet, even though many studies focus on HSP60, we suggest focusing rather on inducible HSPs that are seldom present in non-stressed normal cells. Among these, HSP70 seems to be particularly interesting as it is the most abundant inducible HSP in cancer cells and is also present in the membrane of cancer cells but not in non-transformed cells. In contrast to the common strategy relying on the combination of several cancer biomarkers (Fung et al., 2007; Seigneuric et al., 2009, 2010), HSP70 could allow a detection at different levels: circulating protein, tumor-derived exosomes, and CTCs. Of course, these assays need to be optimized and standardized. Furthermore, their sensitivity and specificity should be addressed. As we recently have shown, another interesting inducible HSP may be HSP110 whose expression can be used for the diagnosis of colon cancer patients presenting micro-satellite instability (Dorard et al., 2011).

Because many HSP studies utilize a small number of samples, there is a real need to coordinate studies and conduct large prospective clinical trials assessing for diagnosis, follow-up, or response to treatment not only HSPs but also other potential biomarkers simultaneously using quantitative methods. One option is the design of surface plasmon resonance techniques measuring several proteins in parallel (Seigneuric et al., 2010) as well as tumor-exosomes and CTCs in biological fluids.

These are new and interesting frontiers worth investigating that may offer the opportunity to detect cancer early danger signals.

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Microsatellite Instability in Colorectal Cancer: Time to Stop Hiding!

Alex Duval, Ada Collura, Kevin Berthenet, Anaïs Lagrange, Carmen Garrido

Colorectal cancer (CRC) is the second cause of cancer-related death worldwide. Surgery constitutes the primary therapy for these tumors, together with chemotherapy that is usually recommended in patients with metastatic primary CRC. Although molecularly distinct entities arising from different physiopathogenic mechanisms - microsatellite (MSI) and chromosomal instability (also called microsatellite stable, MSS) - have been characterized in CRC, there is still no specific therapeutic approach that takes into account disease's molecular heterogeneity [1]. MSI is observed in 10-15% of sporadic CRCs. MSI CRCs displayed particular morphologic features, with greater predilection for the right colon, mucinous histology, low metastatic power, poorer differentiation and higher numbers of tumor-infiltrating lymphocytes. They have been consistently reported to show an improved prognosis and a different response to chemotherapeutic agents. In a recent article in *Nature Medicine*, we have reported the specific mutation of the molecular chaperone HSP110 in MSI CRCs and how the presence of this mutant may constitute a first step towards the understanding of their particular clinical characteristics [2].

It is now well established that MMR deficiency is not in itself a direct transforming event and that the development of these tumors is MSI-driven. This distinctive MSI pathway is characterized by somatic mutational events affecting short coding repeated sequences that, when having an oncogenic effect, provide selective pressure during tumor progression [3]. We showed that a T_{17} mononucleotide repeat located in intron 8 of *HSP110* was systematically mutated in MSI CRC cell lines and primary tumors [2]. The shortening of this repeat in tumor DNA correlated with increased synthesis of an aberrant *HSP110* transcript due to exon 9 skipping, to the detriment of wild-type *HSP110* mRNA. As a result, a truncated HSP110 mutant protein (HSP110DE9) accumulated in MSI tumors. Strikingly, we demonstrated that HSP110DE9 acts as a dominant negative mutant that binds to HSP110 abrogating its chaperone activity and cytoprotective function. In colon tumors, HSPs including HSP110 have been clearly shown to promote cancer cell survival, protecting oncogenic proteins and inhibiting apoptosis [4-6]. It is thus unclear why HSP110DE9 proapoptotic mutant is selected during MSI tumorigenesis. Long, noncoding mononucleotide repeats such as the T_{17} located in *HSP110* intron 8 constitute hot spots for mutations in MSI tumors due to the MMR deficiency. Our hypothesis is that when these mutations are endowed with a biological anti-cancer activity, as it is the case with HSP110DE9, they can represent an Achilles' heel in the MSI-driven tumorigenic process. Further studies are now necessary to determine the exact role of HSP110DE9 during MSI tumor progression and to understand the contribution of HSP110DE9 in the more favorable prognosis of CRC MSI compared to MSS patients.

In vitro, HSP110DE9 expression sensitized colon cancer cells to anticancer agents such as oxaliplatin and 5-fluorouracil, which are routinely prescribed in the adjuvant treatment of patients with CRC [7]. In line with these results, we observed that MSI CRC patients with high HSP110DE9 expression levels who received chemotherapy were all associated with disease-free survival [2]. Therefore, HSP110DE9 levels are likely to constitute a crucial determinant for MSI CRC patients' prognosis and treatment response. Because this mutant protein was expressed at variable levels in these tumors, our findings thus provide evidence for an additional layer of clinical heterogeneity among MSI colon cancers. Additional studies in larger populations are now being performed in order to confirm these results. MSI CRC patients have been recurrently reported to benefit less from 5-FU treatment whereas they seem to show improved response to 5-FU-oxaliplatin (FOLFOX) that constitute today the gold standard of adjuvant chemotherapy in CRC. As Dr. Andrew T. Chan mentions in a recent issue in *Nature Medicine* [8], *"it is fascinating to speculate that such studies might show a lack of response to 5-FU confined to the MSI CRCs with low levels of HSP110DE9"*.

In tumor samples, MSI phenotype can be determined by PCR according to international criteria or by immunohistochemistry studying mismatch repair (MMR) protein expression affecting MLH1, MSH2, MSH6 or PMS2. Our findings highlight that routine evaluation of the MSI phenotype together with investigation of HSP110 status could be of clinical interest in CRC diagnosis. Note worthily, HSP110DE9 is the first HSP mutant identified in a cancer so far. Developing inhibitors of HSP110 that mimic the anti-cancer chemosensitizing effect of HSP110DE9 is also a promising perspective.

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Modulation of normal and malignant plasma cells function by toll-like receptors

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1. ABSTRACT

Toll-like receptors (TLRs) are well known activators of immune responses, but their involvement in the plasma cell (PC) differentiation process remains mostly unknown. This review is focused on the expression and function of TLRs on normal PCs and their malignant counterpart, Multiple Myeloma cells. We report studies that suggest a role for TLR ligands as adjuvants of the humoral immune response through the survival of newly generated immature PCs. On the contrary, TLRs do not seem to be involved in the long-term maintenance of PCs in the bone marrow. Multiple Myeloma cells express a broad range of TLRs, and show heterogeneous responses to different ligands. These double-edged-sword effects are presented and discussed in the context of tumor progression, and as putative therapeutic targets.

2. INTRODUCTION

Vertebrates are frequently attacked by multiple infectious agents throughout their life. To counteract this danger, evolution has selected a panel of receptors that recognize these agents and alert the immune system. Among these receptors, Toll-like receptors (TLRs) play a major role (1). TLRs are type-I transmembrane proteins bearing a cytoplasm Toll/IL-1R homology domain (TIR). They recognize highly conserved specific structures from pathogens (bacteria and viruses) that are named pathogen-associated molecular patterns (PAMPs). So far, 10 and 13 TLRs have been identified in humans and mice respectively. For each TLR, one or several corresponding ligands have been identified, except for TLR10, which is not functional in the mouse. Each TLR has a different specificity in terms of PAMP recognition (2-7). In addition to responding to PAMPs, TLRs also respond to endogenous molecules, which are mostly produced during cellular stress or death as heat shock proteins, products of extracellular matrix degradation, and endogenous RNA or DNA (8-10). They are named danger-associated molecular patterns (DAMP). The engagement of TLRs by their ligands induces various signalling pathways (11). TLRs first dimerize and recruit several TIR-domain-containing adaptor molecules: MYD88, TIRAP, TRIF, and TRAM. MyD88 is used by all of the TLRs except for TLR3, whereas TRIF is used only by TLR3 and TLR4. Activated signaling pathways involve NF-kB, mitogen-activated protein kinases (MAPKs) and PI3k/Akt that lead to cell activation, maturation, type I interferon and inflammatory cytokine production.

Murine and human B cells express several TLRs whose level of expression and responsiveness varies depending on the B-cell subsets (12, 13). *In vitro*, TLR activation of B cells results in the activation, proliferation and differentiation into immunoglobulin-secreting plasma-cells (PCs) (12). Memory B cells have a greater capacity to proliferate and differentiate into PCs after TLR stimulation than do naïve B cells. This has been supported by a study showing that polyclonal activation of human memory B cells through TLRs is essential for the maintenance of serological memory (14). Other *in vivo* experiments support the notion that TLRs are adjuvants that accelerate antibody responses, but are not essential for long-tem humoral responses (15-17). Altogether, these studies shed light on the importance of TLRs in the initial steps of B cell activation. However, B cell differentiation into immature PCs and then matured long-lived PCs is a multistep process that could be influenced by many cytokines and cell-cell interactions (18). A growing body of literature suggests that PAMPs could also target PCs on their way

toward terminal differentiation, and their malignant counterpart that is Multiple Myeloma (MM). The scope of this review is therefore to provide an overview of the literature addressing TLR expression and function in PCs from normal and malignant origin.

3. PLASMA CELLS ARE EFFECTORS OF HUMORAL RESPONSES

3.1. Plasma cell differentiation is a multistep process.

Protective humoral immunity is provided by PCs through their production of antibodies (19, 20). PCs arise by two different routes: one occurring inside the germinal center of secondary lymphoid organs, and one occurring outside the follicle. Several parameters influence the decision to follow one route or the other, including the nature of B cells that are activated (naïve versus memory), the nature of the antigen, and the affinity of the B cell receptor (BCR) for the antigen. For example, in the absence of T cell signaling, memory B cells are the only cells to differentiate into PC upon TLR activation. PCs arising from the germinal center route are generally considered immature and are precursors of long-lived PCs that are able to migrate within the bone marrow and produce a high amount of highly specific antibody. On the contrary, PCs of extrafollicular origin are short-lived, as most of them do not migrate to the bone marrow and these produce only a small amount of antibody. The immature PCs exit the lymphoid organs into the peripheral blood and continue to differentiate into fully mature PCs upon recruitment into an appropriate site of survival (mucosal tissues, bone-marrow or inflamed tissues) (21-23). The differentiation of B cells into immature PCs and then into fully mature antibody secreting cells involves profound molecular changes. Several transcription factors that negatively feed back onto each other are involved in this process (Pax5, Blimp-1, XBP-1 for example) (24, 25). Profound morphological and phenotypical changes also occur during this differentiation. Immature PCs are also called plasmablasts, as they are proliferating cells with low Ig secreting capacities. They are detected in the blood six to eight days after an antigenic challenge. They are also found in secondary lymphoid organs, such as tonsils, but probably at a later stage of maturation (26). Thanks to the study of reactive plasmacytoses that are expansions of plasmablasts retaining the capacity to differentiate into mature PCs, the phenotype of these circulating immature cells has been characterised (CD19+, CD45++, HLA-DR++, CD38++, CD138-). This phenotype switches to CD19+/-, CD45+, HLA-DR+/-, CD38+++, CD138+ upon differentiation into mature PCs (27). Of note, tonsillar plasmablasts have a low expression of CD138. Plasmablasts are short-lived but can be transiently rescued from apoptosis by IL-6, IFN-alpha or plasmacytoid dendritic cells (28, 29).

3.2. Rescuing immature plasma cells from apoptosis in cellular niches

Although long-lived PCs are responsible for sustaining serological memory, they represent a minor fraction of cells within the bone marrow in humans and mice (0.1 to 1% of bone marrow cells) (30). Interestingly this pool of PC is quite constant over a lifespan, despite infections that each potentially generates 104 to 105 new cells (31). The size of the PC population must therefore be tightly regulated. One hypothesis is that newly generated PCs must compete with previously formed PC to access and occupy a limited number of survival environmental niches (32-34). In other words, following infection or vaccination, newly generated PCs are prone to die if they do not find an appropriate niche. At the same time, existing PCs from previous infections are displaced from the bone marrow by these new PCs, circulate in the blood where survival factors are limited, and eventually die. Evidently, this replacement would not result in the eradication of older PCs but would probably affect a minor portion of the PC pool as the repertoire of antigen specificity is maintained over years (20).

What is meant by “long-lived” with regards to PCs? Recent studies have tried to evaluate the duration of the humoral response after the first antigen encounter. It has been shown that long-lived antibody responses after diphtheria and tetanus toxin vaccination can last from ten to twenty years, whereas a response against a live viral infection could last much longer (35, 36). Furthermore, an earlier study estimated the half-live of virus-specific PC in mice as 172 days in the spleen and 94 days in the bone marrow. Both half-lives are quite long compared with the lifespan of the mouse itself (37).

Many soluble factors and cell-cell interactions constitute the bone marrow niche. Stromal cells play an initial role through stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, as PCs express CXCR4 and are attracted by SDF-1 (38). Furthermore, CXCR4 deficient mice have a marked decrease in the bone marrow homing of PCs (39). In addition to bone marrow stromal cells, osteoclasts support the survival of PCs from immature to mature stages *in vitro* (40). Recently, neutrophils have been shown to co-localize with PCs in the bone marrow niche, and strongly support PC survival through the production of IL-6 and APRIL (41). This last report confirms that members of the tumor necrosis factor (TNF) family such as BAFF, APRIL, TACI and BCMA, seem to be involved in the maintenance of long-lived PCs (42, 43). Regardless of the cell to cell interactions, or the cytokine cocktail present in the niche, signalling probably converges on the same target gene, i.e, prdm-1, the gene encoding Blimp-1 (B-lymphocyte-induced maturation protein-1). Blimp-1 is instrumental in the survival of PCs in the bone marrow, as conditional deletion of prdm-1 induces a disappearance of long-lived PC from the bone marrow (44). Of note, besides the many cytokines found in the bone marrow, LPS and CpG-ODNs are also able to induce Blimp-1 expression *in vitro* (45).

PCs therefore appear as a heterogeneous population when characterized over the differentiation process, from their site of origin in secondary lymphoid organs to their site of long-term survival. They depend on microenvironmental niches for their survival and final differentiation. It could therefore be hypothesized that TLR signaling could aid these “PCs-to be” during the course of the infection.

4. MULTIPLE MYELOMA : A MALIGNANT PLASMACYTOSIS

Multiple myeloma (MM) is a fatal plasma cell malignancy that is characterized by excess monoclonal bone marrow plasma cells (PC), osteolytic bone lesions, renal failure and immunodeficiency (46, 47). Malignant PCs secrete monoclonal immunoglobulin (IgG and IgA). In the USA, MM is the second most frequent blood malignancy after non-Hodgkin lymphoma. The disease accounts for about 1 % of all neoplastic diseases and 10% of all hematological malignancies.
In recent years, considerable advances in the genetic and phenotypic characterization of MM have been made (48-51). MM appears as a heterogeneous disease in terms of phenotype, genotype, growth factors and drug responses. The cellular origin of MM PCs is still a matter of debate (52) but the disease always emerges from a state of medullar plasmacytosis often seen in the elderly population, characterized by the presence of serum monoclonal IgG or IgA with no sign of organ failure or bone resorption (53). This premalignant state is known as Monoclonal Gammopathy of Undetermined Significance (MGUS). Numerous kariotic and genetic abnormalities are seen in MM PCs at an early state of disease progression (54). MM is associated with translocations of the heavy chain immunoglobulin gene (14q32) with different oncogenic partners that could be CCND1 (t (11;14)), CCND3 (t (6;14)), MAF (t (14;16)), FGFR3 (t (4;14)) or MAFB t (14;20)). A partial or complete loss of chromosome 13 can also be observed in MM (55).

Thanks to array comparative genomic hybridization, the genetic heterogeneity of MM patients has been classified into 7 groups (56). CD-1 and CD-2 groups are characterized by overexpression of cyclin D1 and D3. The MS and the MAF groups have an overexpression of FGFR3 or MAF respectively. The HY group represents 50% of patients with a hyperdiploid profile. The LB group has a low level of osteolytic lesions and, finally, the PR group is characterized by overexpression of genes associated with proliferation. Secondary late onset translocations and gene mutations can be implicated in the progression from MGUS to overt MM such as MYC (57), NRAS, KRAS and FGFR3 mutations (58).

The bone marrow microenvironnement of MM PC constitutes a cellular niche as it has an essential role in the development, maintenance and progression of the disease (59). Direct interactions between MM cells and bone marrow stromal cells or extracellular matrix proteins promote and sustain MM growth factor secretion together with cell survival signaling pathways. Among many other bone marrow cytokines, IL-6, IGF1, and members of the TNF superfamily such as BAFF and APRIL play a major role (60, 61). In addition to soluble factors, receptors involved in the adherence of MM to the bone marrow niche such as integrins, cadherins, selectins, syndecans also play a major role in the medullar niche (62).

5. TLR EXPRESSION AND SIGNALING IN NORMAL AND MM PLASMA-CELLS

Tonsillar PCs express all TLR (except 10) at levels comparable to tonsillar naïve and memory B cells (63). This expression does not seem to be altered after exiting secondary lymphoid organs as a similar pattern has been found in peripheral blood plasma cells. PCs finally mature in the bone marrow where they become long-lived PCs (37). It has been shown that they express either only significant levels of TLR1, RP105, MD-1 and MD-2 (64) or of TLR1, 5 and 9 (65). These studies (as for most studies on TLR expression) suffer from a lack of reliable protein detection. Indeed, commercially available TLR antibodies fail to give satisfactory results in human cells. Therefore, once available, reevaluation of TLR expression by other means than RT-PCR might help to definitively address the question of the TLR expression profile during PC differentiation. Fortunately, a recent study addressed the question of the level of TLR9 mRNA that correlates with a significant cellular response. This quantitative RNA approach circumvents the problems of protein detection (66). However, the authors show that, above the defined threshold of TLR9 expression, the magnitude of response to CpG-ODN is dependent on tumor cell type.

MM primary cells and human myeloma cell lines (HMCLs) express a broad range of TLR (64, 65, 67-70). A study of primary MM cells from 414 patients at diagnosis revealed an almost universal expression of TLR9, and around 30% expressed TLR3, 5 and 7. A comparison of TLR levels of expression by real-time PCR showed that TLR2, 4 and 9 were significantly higher expressed in bone marrow mononuclear cells from MM patients than in control donors (65). Even if the expression on PCs was not directly addressed, this increase suggests that cells of MM bone marrow have good potential to respond to PAMPs. In addition to TLRs, their signaling proteins seem to be expressed in the majority of HMCLs, ie, MYD88, TRIF, and TRAF6 (personal data and (68, 71). TRAF3 expression, however, seems less frequent as it is mutated or deleted in 12,3% of primary MM cells and 17% of HMCLs (72). This lack of expression could impact the growth of MM cells as TRAF3 is a known inhibitor of the non-canonical NF-kB pathway, and could participate to sustain NF-kB signaling. Furthermore, TRAF3 negative MM cells could be more sensitive to TLR ligands. This is supported by the finding that TRAF3 transgenic mice show an increased response to antigen and TLR agonists, which evolves over time into plasmacytosis and hypergammaglobulinemia (73). Distribution of the TLR expression pattern appears heterogeneous when the molecular classification of patients defined by Zhan described above is addressed (Table 1). Indeed, patients with a favorable outcome, such as hyperdiploïd patients, express TLR3 and TLR7 more frequently compared to other groups, but lower levels of TLR4 and 9. Inversely, TLR9 and 4 are overexpressed by MS and MAF groups that both have a poorer prognosis. As Maf is a transcription factor, it could be of interest to determine its involvement in TLR4 expression. On HMCLs, a pattern similar to primary MM cells is observed. Indeed, TLR1, 7, and 9 are the most strongly expressed (96%, 68% and 64% of cell lines respectively). TLR3 and 4 are expressed in nearly half of the HMCLs, whereas TLR2 and 8 are only detected in 28% and 24% of the cell lines, respectively (personal data, n=25).

6. COULD MM PLASMA CELLS ENCOUNTER TLR LIGANDS ?

In MM, normal PC differentiation and survival is blunted due to competition with MM PC for the medullar niche. As a consequence, the level of normal IgG is low and patients are highly susceptible to recurrent and persistent bacterial, fungal, and viral infections. Next to general organ failure, infections are a main cause of death for MM patients (46). A retrospective epidemiological study based on 4641 MM and 2046 MGUS patients has shown that the risk of developing the disease is significantly increased when patients have a history of multiple infections such as pneumonia, hepatitis, meningitis, septicemia, herpes zoster, and poliomyelitis. Risk was also associated with influenza infection, but only for white men (74). Furthermore, a history of pneumonia in the 5 years preceding MGUS appearance has been shown to be a predictor of MGUS risk (75). This finding suggests that infectious agents by themselves, or infection-associated inflammation could be potential triggers for MGUS and/or MM development.

During the course of the disease, several pathogens are associated with MM, such as Gram-positive bacteria (*Streptococcus pneumonia, Staphylococcal* infections) and Gram-negative bacteria (*Pseudomonas, Haemophilus influenzae, Escherichia coli*) as well as invasive fungal infections. Numerous sources of PAMP are therefore present in or even associated with MM. One should not forget, however, that even in the absence of a clear correlation of MM progression with a particular infectious agent, the bone marrow environment is still a soup of DAMP. Indeed, MM cells deregulate the balance of bone resorption/formation by inhibiting osteoblastogenesis and promoting osteoclastogenesis. The consequence is a dramatic bone resorption that impairs the patient's quality of life, increasing the release of extracellular matrix proteins, fibronectin, collagen and inflammatory mediators (76). Many of these microenvironment components are described as TLR agonists (77).

High Mobility Group Bex1 (HMGB1) protein is the most abundant chromatin-associated non-histone protein expressed in all nucleated eukaryotic cells. It is also released in the extracellular medium by macrophages after TLR stimulation or during cell death (78). HMGB1 is described as a TLR4 agonist and a TLR3, 7 and 9 co-ligand (79, 80). HMGB1 accelerates the delivery of CpG-ODNs to its receptor (81). Interestingly it is released by bone cells such as osteoblasts and osteoclasts in the bone marrow environment. HMGB1 could therefore act as a co-factor for TLR ligand recognition in the MM niche (82). Furthermore, soluble CD138, a molecule belonging to the heparan sulfate family, is a potent TLR4 agonist, and is highly expressed and released by MM PCs. Of note, the soluble CD138 level correlates with poor prognosis (83).

In conclusion, despite the heterogeneous expression of TLR on MM cells, it is highly probably they will encounter a PAMP or DAMP for which they bear the corresponding TLR.

7. FUNCTION OF TLR IN NORMAL PLASMA CELLS

The strong expression of multiple TLR observed on tonsillar and peripheral PCs suggests that they could respond to a broad range of ligands. However, only a few studies have addressed this question, and it remains crucial to have a complete understanding of the mechanism of humoral responses for vaccine design. PCs located in the tonsils or plasmablasts circulating in the peripheral blood are phenotypically and functionally different from the long-lived bone marrow PCs. This discrepancy is also observed for TLR responses. Indeed, PCs from tonsils and peripheral blood have an increase of total Ig synthesis and secretion upon TLR triggering (63). TLR1/2, 3, 5 and 9 are mainly involved in Ig secretion in tonsillar PCs. TLR1/2 ligands exclusively increase intracellular IgM expression and secretion. TLR3 (poly (I:C)) and 9 ligands (CpG-ODNs) increase IgG expression from tonsillar PCs, whereas TLR7 ligand increases IgG expression only in peripheral blood PCs (63). In addition, our personal data suggest that plasmablasts have increased survival and total immunoglobulin secretion in the presence of TLR9 ligands (unpublished data). On the contrary, a study of normal bone marrow mononuclear cells (as opposed to purified PCs) has shown a lack of response (65). Neither an increase of CD138+ cells (a marker of mature PCs), nor a decrease in apoptosis was detected in the presence of TLR1/2 ligand (Pam3), TLR4 ligand (LPS) or TLR9 ligand (CpG-ODNs). These results are in agreement with an absence or a low TLR expression on mature PCs.

Therefore, TLR expression and functions seem to differ accordingly to their developmental stage. Fully matured and long-lived PCs are quiescent cells that do not seem to be affected by infections or PAMPs as plasmablasts are. They are either less sensitive, or completely insensitive to pathogen-derived signals of activation, and their continuous antibody secretion is not altered. This lack of sensitivity is not a hallmark of bone marrow cells, as TLR ligands directly induce human and mouse hematopoietic stem cells to differentiate towards myelopoïesis (84-86).

8. FUNCTION OF TLR IN MULTIPLE MYELOMA CELLS

8.1. TLR on MM cells as protumoral effectors

Can we consider TLRs as receptors for protumoral ligands (either PAMPs or DAMPs) present in the bone marrow microenvironment? In other words, can MM cells hijack the TLR machinery for their own benefit? This question is worth addressing due to the significant expression of TLR and their signaling molecules found in MM cells. This question can be considered on three different levels: first cell growth, second apoptosis, and finally immune escape.

Proliferation of several HMCLs and primary MM cells can be increased by most of the TLR ligands when receptors are present (64, 67). Most studies, however, focus only on ligands of TLR4 and 9. The increase in proliferation is mainly mediated by an autocrine IL-6 loop that can substitute paracrine IL-6. Indeed, IL-6 is a major proliferation and survival factor for MM cells (87, 88) and circulating IL-6, as well as circulating soluble IL-6R, was found to increase during MM progression (89). In MM, IL-6 originates from the bone marrow microenvironnement. Therefore, this supplementary contribution could enhance the pathogenic potential of the milieu where MM cells emerge, and facilitate their growth or maintenance in the case of an altered niche (as a treatment consequence for example). Autocrine TLR-induced or paracrine IL-6 is not the only cytokine that stimulates growth, as Insulin growth factor -1 (IGF-1) besides IL-6 (either autocrine or paracrine), is also a major cytokine stimulating MM cell growth. However, so far no increase in expression of IGF-1 has been reported after TLR triggering. This could be worth investigating, as TLR2 and 4 agonists can induce an IL-6 independent proliferation of the RPMI-8226 HMCL (64).

Protection against various apoptotic conditions is also a feature of TLR stimulation. Serum or IL-6 deprivation is, accordingly to the IL-6 induction, compensated by TLR4 and 9 (67). Drug-induced apoptosis by dexamethasone or adriamycin (both effective drugs for MM) is partially or totally inhibited by pretreatment of HMCLs by LPS or CpG-ODNs (67, 90). In addition to the action of chemotherapy several effectors of the immune system (CD8+ T cells, gamma-delta T cells and NK cells) have the potential to kill primary MM cells or HMCLs in both antigen-dependent or independent mechanisms (91-93). However, due to the age of MM patients (the median age at diagnosis is 65) and their immune deficiency (for example MM dendritic cell dysfunction (94), and regulatory T cell accumulation (95)) that is somehow common to most cancer patients, immune effectors from MM patients have limited action towards malignant cells. To boost vaccination strategies with myeloma-derived antigen, adjuvant therapies using bacterial extracts, such as BCG (TLR2, and 4 ligands) streptococcus preparation (TLR4 ligand), imiquimod (TLR7 ligand), or CpG-ODNs (TLR9 ligand) have been proposed. Synthetic TLR9 ligands CpG-ODNs are currently the most used adjuvants in cancer therapy. To enhance their half-life from a few minutes to two days (96) and to enhance affinity to TLR9, CpG-ODNs are chemically modified to replace the native phosphodiester backbone by a phosphorothioate (PS) backbone. However, this modification has significant drawbacks, because it induces unexpected TLR9-independent side effects (69, 97, 98). Indeed, synthetic PS-modified CpG-ODNs inhibit the killing of various TRAIL-R2 (DR5) sensitive tumor cells (MM, breast carcinoma and colon carcinoma) through a TLR9-independent, but PS-dependent binding to TRAIL-R2 ligand. Moreover, this inhibition reduces the TRAIL-mediated cytotoxicity of NK cells, and could therefore interfere with the clinical efficiency of a TLR9 agonist based adjuvant. Similarly, the apoptosis induced by the bone marrow-originated bone morphogenetic protein-2 (BMP-2) is inhibited by PS-modified CpG-ODNs in a TLR9 independent manner (98). Encapsulation of CpG-ODNs, which avoids unspecific binding with unexpected consequences, could be used, especially in clinical trials.

The proliferation and cytotoxicity of NK cells and T lymphocytes can be inhibited by immunoregulatory molecules expressed on or released by tumor cells such as B7-H1 and B7-H2 (99). B7-H1 is a ligand for PD-1, and is mainly induced by IFNg in normal cells. Both molecules are overexpressed in MM cells, but not in plasma cells from MGUS patients. TLR4 agonists significantly enhance B7-H1 and B7-H2 expression through a MyD88/TRAF6-dependent pathway involving MAPKs ERK1, 2 (68). Accordingly, LPS and IFN-gamma inhibit both CTL generation and T lymphocyte proliferation with the same capacity, suggesting that B7-H1/2 expression could be a responsible for the effect. Therefore TLR4 and 9 signaling, in addition to their classical adjuvant function on the immune system, could have an unwanted effect on tumors leading to immune evasion.

Several signaling pathways could mediate the proliferative/survival signals triggered by TLR ligands. MM cells have a strong NF-kB activity, owing to activating mutations of NIK, NF-kB1, NF-kB2, TRAF2, TRAF3, or CYLD in 15-20% of the myeloma cells (72), and to NF-kB-inducing cytokines produced by the microenvironement, such as TNF-alpha, Baff or RANKL (100, 101). TLR ligands could be added to this list, acting through either the MYD88 or TRIF-dependent pathway. Indeed, NF-kB activation has been shown in several HMCLs in response to TLR3, TLR4 or TLR9 ligands (65, 66, 70). IL-6 production and IL-6-induced proliferation depend on NF-kB in most HMCLs (65, 70). MAP kinases Erk1, 2 and JNK have also been implicated in MM proliferation (90), or in the overexpression of B7-H1 (68).

8.2. TLR on MM cells as therapeutic targets

We have learned from seminal works on dendritic cells that responses to TLRs are heterogeneous, depending not only on TLR structure and signaling adaptators diversity, but also on the subsets of dendritic cells (102). A similar model can be applied to non immune cells and to cancer cells. In addition to pro-survival signals, which reflect mainly NF-kB and MAP kinase-dependent transcription of genes, apoptotic messages can be generated upon TLR2, 3, 4, 7 or 9 triggering in various cancer cell types (103). TLR3, which signals through a MYD88-independent/TRIF dependent pathway, is the best characterized. The synthetic TLR3 agonist (poly (I:C)) is known to directly induce apoptosis in several solid cancer cells and has been used in clinical trials as an immune activator. This dual anti-tumoral characteristic turns it into a particularly interesting therapeutic target (104). TLR3 is expressed in nearly 50% of HMCLs, and 25% of primary myeloma cells. We found that poly (IC) induces growth inhibition or apoptosis in three out of six TLR3 positive HMCLs studied. In accordance with the results highlighted in HMCLs (64, 70), a decrease of primary myeloma cell viability was observed in two out of four patient samples after *in vitro* poly (IC) stimulation (personal data). TLR3-dependent apoptotic process in MM was found to be linked to the production of an autocrine IFN-alpha loop, controlled by early p38 MAPK activation (70). Involvement of p38 MAPK activation in IFN-alpha secretion induced by TLRs seemed to be a conserved pathway in MM and pDCs at least (105). Moreover, poly (IC)-induced apoptosis in MM is dependent on caspases 3, 8, and 9. Implication of caspases in poly (IC)/IFN-alpha-dependent apoptosis is in accordance

with the results described in melanoma or breast cancer cells (104). TRAF3, a major regulator of type I IFN, does not seem to be involved in the poly (IC) effect as it is mutated and non-functional in some HMCLs induced in apoptosis.

However, we observed that poly (IC) also induced NF-kB activation in all TLR3 positive cell lines, independently of IFN-alpha secretion. In the presence of IFN-alpha, NF-kB could not overcome the apoptosis signal. This suggests that MM cell fate after TLR triggering will depend on the balance of survival versus apoptotic signals. Therefore, to shift the balance in favor of apoptosis, a possible therapeutic approach would be the use of a TLR3 ligand to stimulate the immune system, together with inducers of MM cell death. Activation of the immune system could also be achieved by targeting TLR9 on the surface of plasmacytoid dendritic cells from patients. Indeed, MM-plasmacytoid dendritic cells exhibit altered function and reduced ability to induce T-cell proliferation, which can be reversed by stimulation with CpG-ODNs (106).

9. CONCLUSION

At any one time, a body's population of PCs is heterogeneous, with regard to their localization (mucosa, secondary lymphoid organs, bone marrow), their origin (follicular or extrafollicular), their half-life, and their stage of maturation (plasmablasts, or mature PCs). Similarly, TLR expression and function seem to follow the differentiation process as plasmablasts or PCs from tonsils, but not mature bone marrow PCs, respond to TLR stimulation. Immature PCs and PCs located within sites of B cell activation or pathogen encounter could be therefore still be activated by surrounding molecules from the pathogen during the course of an infection. Later on, once the immune system has been fully mobilized and PCs have found a molecular niche to persist for years in the absence of antigen, the effect of TLRs is no longer needed for the persistence of the serological memory. This review of the literature suggests that TLRs act as an adjuvant of humoral immune responses not only by targeting B cells, but also plasmablasts and immature PCs.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the expansion of autoreactive plasmablasts and PCs (107, 108). Interestingly, many DAMP are present in SLE patients, and endogenous TLR7 and TLR9 ligands have been shown to promote the disease through dendritic cell stimulation (109). IFN-alpha is a major cytokine in SLE physiopathology and a survival factor for PCs. In this context it is of interest that TLR3 is expressed on peripheral blood PCs, and that its stimulation can initiate the production of IFN-alpha by MM PCs. This raises the question if this cytokine production is also a characteristic of normal and SLE plasmablasts. Given this review of the literature, TLRs ligands might participate directly in SLE, contributing to the survival and expansion of the effectors of the disease.

The capacity to respond to TLRs ligands is conserved by MM cells, the malignant counterpart of PCs. MM cells express a broad and heterogeneous range of TLRs associated with various responses *in vitro* (Figure 1). Heterogeneous responses to TLR ligands are also observed in other hematological and solid malignancies, resulting in either pro-tumoral effects or induction of apoptosis. Thus, cancer therapies using TLRs ligands should first identify predictive biomarkers of apoptotic responses to TLR ligands. Furthermore, the uncoupling of the pro-tumoral effects of TLR ligands from their adjuvant effects on the immune system is mandatory. This could be achieved by a simultaneous neutralization of MM growth factors or survival pathways. Is there a role for PAMP or DAMP in the emergence of MM? Due to the lack of *in vivo* data available in animal models, we are restricted only to speculate on this. A select few epidemiological studies, together with the appearance of strong TLR expression and co-receptors during the MGUS to MM transition, support this hypothesis. Given their immune adjuvant function during infection or vaccination, TLR ligands could therefore perhaps also act as "tumoral adjuvants" in the context of malignant clone emergence. In this scope, the study of TLR expression and function in MM stem cells is worth being investigated.

10. ACKNOWLEDGEMENTS

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Abbreviations: TLRs: Toll-like receptors. MM: Multiple Myeloma. PC: Plasma-cell. TIR : Toll/IL-1R homology domain. PAMP : pathogen-associated molecular patterns. dsRNA : double stranded RNA. LPS : lipopolysachcaride. CpG : unmethylated 2'-deoxyribo (cytidine-phosphtae-guanosine) . DAMP: danger-associated molecular patterns. MAPKs: mitogen-activated protein

kinases. SDF-1: stromal cell-derived factor-1. TNF: tumor necrosis factor. Blimp-1: B-lymphocyte-induced maturation protein-1. MGUS: Monoclonal Gammopathy of Undetermined Significance. HMCLs: human Myeloma cell lines. HMGB1: High Mobility Group Box1.

Key Words: Toll-like receptor, Plasma-cell, Plasmablast, Bone-marrow, Multiple myeloma, Humoral, Review

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Figure 1. Overview of the consequences of TLR activation on MM cells. Protumoral effects on MM cells have been observed with ligands of TLR2/6, TLR4, TLR5, TLR7/8, and TLR9. These ligands trigger signaling pathways as MAPK ERK1/2 and NF-kB. Cellular outcome are proliferation, resistance to apoptosis and immune escape. On the contrary, on some primary MM cells or HMCLs, TLR3 ligand induces IFN-alpha secretion through MAPK p38 signaling pathway and induces cell death. The apoptosis is observed despite NF-kB activation. This finding suggests that IFN-alpha production is a molecular determinant of the TLR triggering outcome.

Table 1. Heterogeneous expression of TLR3, 4, 7 and 9 on primary myeloma cells**.** Expression of TLR3, 4, 7 and 9 on MM primary cells with respect to molecular classification as defined by Zhan et al.: PR: proliferation, LB: low bone disease, MS: MMSET, HY: hyperdiploid, CD: cyclinD, MF: MAF. Median and range represent TLR mRNA expression in relative units. p values are determined using CHI2 test for positive samples expression and using Wilcoxon test for expression level. Significant over-expressions and underexpressions are represented in bold and italic respectively. NS = non-significant. Data can be accessed on the website http://amazonia.transcriptome.eu/. Reprinted from leukemia Research, 34 (12), David Chiron, Gaetan Jego, Catherine Pellat-Deceunynck, Toll-like receptors: Expression and involvement in Multiple Myeloma, page 1545-50, Copyright (2010), with permission from Elsevier

Running title: Plasma cells and TLR

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NOUVELLE

Mutation d'HSP110 dans les cancers colorectaux

Le paradoxe du chaperon qui ne protège plus

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Mutation de HSP110 dans les cancers colorectaux

Les protéines chaperons ou HSP (*heat shock proteins*) sont essentielles à la cellule. Elles se comportent comme des chaperons ou « capuchons moléculaires » pour d'autres protéines cellulaires, intervenant ainsi dans de nombreux processus biologiques. Chez les mammifères, il existe cinq principales familles de protéines HSP, classées en fonction de leurs poids moléculaires (HSP100, HSP90, HSP70, HSP60 et les petites HSP) (pour revue, *voir* [1]).

Certaines de ces protéines HSP sont surexprimées par les cellules tumorales, ce qui leur permet ainsi de s'adapter aux conditions environnementales qu'elles ont à affronter au cours du développement du cancer chez le patient ; l'expression accrue de ces HSP protège par exemple les cellules tumorales de la mort cellulaire, notamment celle qu'induisent les drogues utilisées lors du traitement des patients par chimiothérapie. Plusieurs inhibiteurs de protéines chaperons pourraient être inclus dans l'arsenal thérapeutique anticancéreux et sont actuellement testés dans des essais cliniques chez l'homme.

En collaboration avec l'équipe de Carmen Garrido, l'équipe d'Alex Duval a identifié la mutation d'un des gènes de la superfamille des HSP, le gène *HSP110,* dans des cancers colorectaux [2]. La protéine mutante perd plusieurs domaines protéiques essentiels à son activité. Elle se lie à la protéine HSP110 normale et l'empêche par là même de jouer son rôle de chaperon dans la cellule. Les cellules tumorales exprimant la protéine mutante sont fragilisées et montrent en particulier une sensibilité accrue aux chimiothérapies prescrites aujourd'hui dans le traitement des patients atteints de cancer du côlon, comme le 5-fluorouracile ou l'oxaliplatine. La protéine mutante est délocalisée dans le cytoplasme, contrairement à la protéine HSP110 sauvage dont la localisation est à la fois cytoplasmique et membranaire *(Figure 1)*.

Mutation de HSP110 : un marqueur prédictif de la réponse thérapeutique

Sur un plan clinique, nous avons observé que la protéine mutante était présente à des taux variables chez 100 % des patients qui souffraient d'une forme particulière de cancer colorectal (CCR). Il s'agit des tumeurs du côlon appelées MSI (pour *microsatellite instability*), qui représentent 20 % environ de l'ensemble des CCR chez l'homme. De manière parfaitement concordante avec les résultats acquis *in vitro* dans des cultures de cellules tumorales, nous avons observé que les patients dont les tumeurs expriment fortement la protéine mutante (35 % des malades porteurs d'un CCR MSI environ) répondent très favorablement à la chimiothérapie puisqu'aucune rechute de la maladie n'est observée chez eux. À l'inverse, les patients qui expriment la protéine mutante à des taux plus

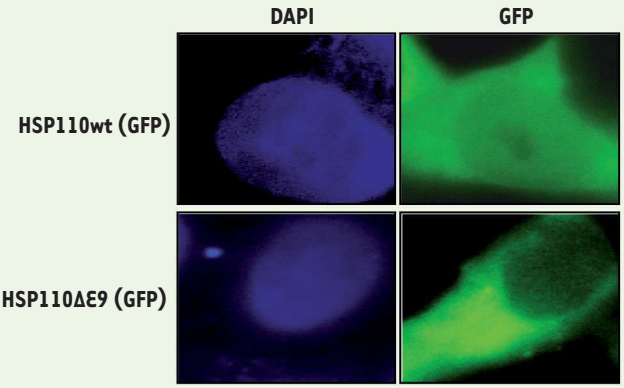

Figure 1. ***Expression de la protéine chaperon HSP110 dans la cellule tumorale colique.*** La protéine sauvage (Hsp110wt) présente une expression nucléaire et cytoplasmique. La protéine mutante (HSP110ΔE9) présente une expression aberrante, restreinte au cytoplasme. GFP : *green fluorescent protein* ; DAPI : marqueur des noyaux.

faibles (65 % des patients porteurs d'un CCR MSI) répondent moins favorablement au traitement et leur maladie récidive. Ces derniers résultats sont particulièrement intéressants sur le plan clinique ; ils attestent en effet que la connaissance du statut dans la tumeur de la protéine HSP110 (forte ou faible expression) permet de prédire la réponse au traitement du patient. Celle-ci étant facilement déterminée en clinique, la prise en charge thérapeutique devrait donc être améliorée. C'est d'autant plus important que le cancer du côlon, un des cancers les plus fréquents dans le monde, représente la deuxième cause de mortalité par cancer chez l'homme, et que les patients bénéficiant d'une chimiothérapie sont ceux qui souffrent d'une forme grave et malheureusement fréquente de la maladie (tumeur invasive associée à des métastases locorégionales, au niveau ganglionnaire) [3]. À plus long terme, la découverte de nouveaux composés qui seraient capables de mimer l'effet chimiosensibilisant de la protéine HSP110 mutante dans la cellule cancéreuse constituerait une avancée significative dans le traitement du cancer.

Une mutation inattendue dans un contexte tumoral

Les cellules tumorales MSI [4-6] présentent un phénotype d'instabilité génomique particulier, consécutif au défaut fonctionnel du système MMR (*mismatch repair*), qui se caractérise par l'accumulation de nombreuses altérations dans les séquences répétées du génome, ou microsatellites [7, 8]. Il semble que le défaut d'expression du gène *HSP110* dans ces tumeurs est consécutif à l'instabilité d'une séquence microsatellite de grande taille (répétition T_{17}), localisée dans un intron et dont la mutation provoque un épissage aberrant par saut de l'exon 9 [2]. L'expression d'une protéine mutante comme la protéine HSP110ΔE9 est un événement *a priori* surprenant puisque son action est délétère pour les cellules tumorales (rôle proapoptotique, effet chimiosensibilisant). Notre hypothèse pour expliquer la survenue fréquente d'un tel événement dans le cancer est que le mécanisme qui en est à l'origine, la délétion du microsatellite intronique T_{17}, est probablement inévitable dans des cellules tumorales déficientes en MMR. En effet, ces clones déficients en MMR, dont l'index mitotique est souvent très élevé, ne sont pas en mesure de réparer les erreurs de réplication qui surviennent inéluctablement et à haute fréquence au niveau de telles répétitions génomiques introniques de grande taille. Cette hypothèse demande à être confirmée afin de percer le mystère de l'expression paradoxale du chaperon qui ne protège plus. ◊

Mutation of HSP110 in colorectal cancer: the chaperone paradox

CONFLIT D'INTÉRÊTS

Les auteurs déclarent n'avoir aucun conflit d'intérêts concernant les données publiées dans cet article.

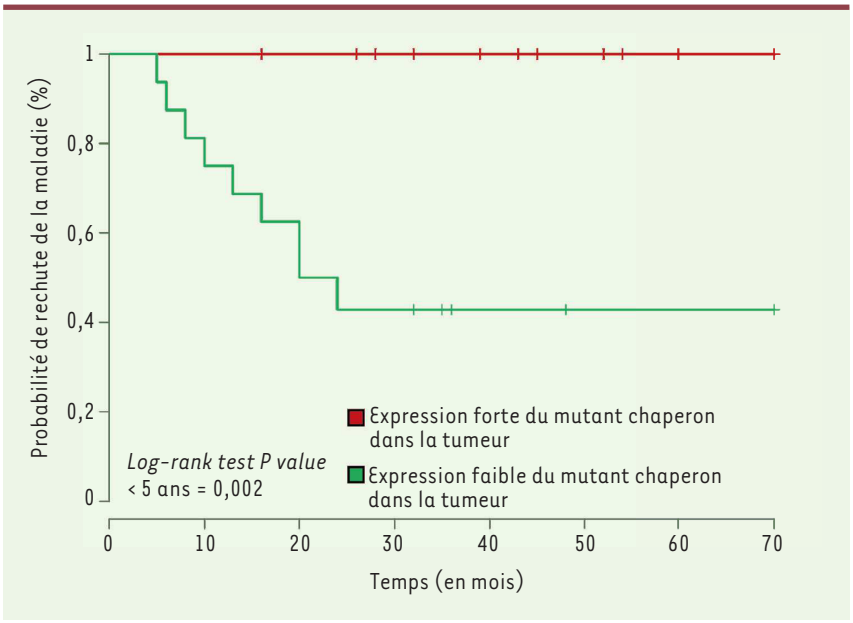

Figure 2. ***Impact de l'expression du mutant chaperon HSP110ΔE9 dans la tumeur sur la réponse à la chimiothérapie des patients atteints de CCR MSI.*** L'expression forte du mutant HSP110ΔE9 dans la tumeur protège d'une rechute de la maladie après traitement par chimiothérapie.

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