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LOCALLY EXPRESSED MELANOCORTIN RECEPTORS MODULATE HUMAN MACROVASCULAR REMODELING

BIO/14

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Summary

ABBREVIATIONS

153N-6 = MC1R antagonist

ACTA2 = α-Smooth-Muscle Actin gene

ACTH = Adrenocorticotropic hormone

AgRP = Agouti-related protein

ASIP = Agouti signaling protein

AUC = area under curve

Ca^{2+} = Calcium

cAMP = Cyclic adenosine mono phosphate

CNN1 = Calponin gene

CNS = Central nervous system

CREB = cAMP response element-binding

CVD = Cardiovascular disease

DES = Drug-eluting stent

dPCR = digital PCR

EC = Endothelial cell

ECM= Extracellular matrix

EL = Extracellular loop

GPCR = G protein-coupled receptor

GRK = G protein-coupled receptor kinase

HAoEC = Human aortic endothelial cell

HAoSMC = Human aortic smooth muscle cell

HCAEC = Human coronary artery endothelial cell

HDMEC = human dermal microvascular endothelial cell

HMEC-1 = transformed human dermal endothelial cell

HSE = heat shock response element

HSF1 = Heat shock factor 1

HSP27 = Heat shock protein 27

ICC = Immunocytochemistry

IHC = Immunohistochemistry

IP_3 = Inositol triphosphate

JKC 363 = MC4R specific antagonist

MC(1-5)R = Melanocortin (1-5) receptor

MCS = Melanocortin system

MK2 = MAPK activated protein kinase 2

MSG-606 = MC1R specific antagonist

MSH-OE = transgenic mice overexpressing MSH

NF-kB = Nuclear factor-kB

NKX2.5 = NK2 Homeobox-5

NO = Nitric oxide

ORF = open reading frame

p38MAPK = p38 mitogen-activated protein kinase

PC = Pro-hormone convertases

PDGF = platelet-derived growth factor

PKA-C = protein kinase A-C

POMC = Proopiomelanocortin

qRT-PCR = Quantitative real time PCR

SERCA = sarco/endoplasmic reticulum Ca^{2+}ATPases

SM22α = Smooth-Muscle-22-α

SMC = Smooth muscle cell

SM-MHC = Smooth muscle myosin heavy chain

SOX9 = SRY-Box 9

TGLN = SM22α gene

TM = Transmembrane

VEGF = Vascular endothelial growth factor

WB = Western blot

α-(β-γ)-MSH = α-(β-γ)-melanocyte stimulating hormone (MCR agonist)

α-SMA = α-Smooth-Muscle Actin

ABSTRACT

Background: Vascular remodeling is a pathological process central to many cardiovascular diseases (CVDs). Vascular injury promotes a complex interplay between distinct cell populations in the vessel wall that results in endothelial dysfunction and local inflammation, which in turn stimulate vascular smooth muscle cell (SMC) proliferation and extracellular matrix (ECM) deposition, leading to neointimal thickening and stenosis. Different signaling pathways, including the melanocortin system (MCS), modulate vascular inflammation and oxidative stress. The MCS refers to a set of hormonal and paracrine signaling pathways that include five G protein-coupled receptors (GPCRs), peptide agonists derived from the post-translational processing of the proopiomelanocortin (POMC) prohormone, ancillary proteins, and endogenous antagonists. This system regulates a remarkably diverse array of physiological functions and host reactions. Preclinical investigations indicate that activation of certain melanocortin receptor (MCR) subtypes, primarily MC1R, could be a novel strategy to control inflammatory disorders. A local MCS has been described in endothelial cells (EC) of the cutaneous and cerebral microcirculation. The melanocortin alpha-melanocyte stimulating hormone (α-MSH) has been shown to modulate blood vessel tone by enhancing nitric oxide (NO)-cyclic-guanosine mono-phosphate dependent relaxation responses through endothelial MC1R. Besides, treatment with MCR agonists was able to prevent the development of vascular dysfunction and attenuate plaque inflammation in pre-established atherosclerosis. We sought to investigate whether human macrovascular ECs and SMCs express locally any MCR and what function MCRs exert.

Methods: Expression of MCS components in primary human aortic (HAoEC), coronary artery ECs (HCAEC) and human aortic smooth muscle cells (HAoSMC) were assessed by real-time PCR (qRT-PCR), western blot (WB), immunocytochemistry (ICC) and immunohistochemistry (IHC). Intracellular cyclic adenosine-mono-phosphate (cAMP) concentrations were measured by an ELISA assay. Intracellular EC calcium (Ca^{2+}) concentrations were analyzed using a fluorescent Ca^{2+} indicator. Gap closure assays were used for assessing *in vitro* cell migration of ECs and SMCs. SMC transmigration was assessed by a transwell migration assays along a chemoattractant gradient (chemotaxis), and proliferation by incorporation and measurement of a nucleoside analog in newly synthesized DNA. Genome-wide gene expression profiling was performed in migrating HAoECs and HAoSMCs after stimulation with α-MSH in time-course experiments, using microarrays and RNA sequencing, respectively. SMC phenotypic changes and associated biomarkers were visualized and measured by ICC and qPCR.

Results: We showed that primary human aortic ECs and SMCs express functionally active MCRs. ECs express MC1R, but not other MCR subtypes nor the precursor hormone POMC. MC1R engagement with α-MSH, its highest-affinity natural ligand, accelerates cell migration in an *in vitro* directional migration assay. This was associated with enhancing in Ca^{2+} signaling and inhibition of cAMP elevation. Time-course genome-wide expression analysis in ECs undergoing directional migration assay allowed identifying dynamic co-regulation of genes involved in the extracellular matrix-receptor interaction, vesicle-mediated trafficking, and metal sensing: all these pathways have a well-established influence on EC motility.

SMCs express MC1R and MC4R, but not POMC. *In vitro* stimulation with α-MSH promoted human aortic SMC differentiation from a synthetic to a contractile phenotype with a spindle-shape conformation. α-MSH promoted significant human aortic SMC elongation with peak effects at 6-12 h after treatment. Stimulation with α-MSH enhanced gene and protein expression of α-Smooth-Muscle Actin (α-SMA) and Smooth-Muscle-22-α (SM22α), which are markers of the contractile phenotype. MCR activation by α-MSH slowed down migration both in the gap-closure assay (peak effect at 9-12 h) and in the transwell migration assay. We confirmed that MC1R is involved in this process because its inhibition with a specific antagonist (MSG-606) restored the normal migration rate. Furthermore, MCR activation slightly decreased HAoSMC proliferation. We then investigated which pathways were activated downstream the receptor in migrating SMC, by analyzing the transcriptome and specific phosphoproteins. We found a negative modulation of the p38 mitogen-activated protein kinase (p38MAPK)/heat shock factor 1 (HSF1) pathway along with a significant reduction in p38-MAPK phosphorylation, which likely is the mechanism of action underlying MC1R-related modulation of SMC migration.

Conclusions: These results provide evidence of a novel function of peripherally expressed MCRs, *i.e.* regulation of EC and SMC motility, ultimately contributing to vessel homeostasis. In addition, this is the first study that seeks to uncover the possible influences of MCRs expressed in macrovascular arteries on the pathophysiology of vascular remodeling. Indeed, results from this study suggest novel, potentially promising therapeutic targets for prevention and healing of vascular remodeling: targeting the MCRs may result in controlling vessel inflammation, healing endothelial dysfunction, and inhibiting SMC phenotypic switching.

RIASSUNTO

Introduzione: il rimodellamento vascolare è un evento comune in molte patologie cardiovascolari. Il danno vascolare attiva complesse interazioni tra le cellule all'interno della parete del vaso che portano a disfunzione endoteliale, infiammazione locale, proliferazione e migrazione delle cellule muscolari lisce (SMC) e deposizione di nuova matrice extracellulare (ECM), causando stenosi e ispessimento della tunica intima o neointima. Diverse vie di segnalazione controllano l'infiammazione e lo stress ossidativo dei vasi, tra cui il sistema melanocortinico (MCS). Il MCS è un complesso sistema ormonale composto da: 5 recettori (MCR) associati a proteine G (GPCR), peptidi derivati dal processamento post-traduzionale del pro-ormone pro-opiomelanocortina (POMC), proteine ancillari e antagonisti endogeni. Questo sistema regola numerose e varie funzioni fisiologiche e risposte infiammatorie. Indagini precliniche indicano che l'attivazione di alcuni MCR, in particolare di MC1R, possa essere una nuova strategia per controllare le patologie infiammatorie. Un MCS attivo è stato descritto a livello locale nelle cellule endoteliali (EC) del microcircolo cutaneo e celebrale umano. Nel topo, l'ormone melanotropo alpha (α-MSH) agendo sul MC1R endoteliale modula il tono arterioso aumentando il rilassamento vascolare indotto dall'ossido nitrico (NO). Inoltre, il trattamento con agonisti dei MCR previene lo sviluppo di disfunzione vascolare e attenua l'infiammazione a livello della placca in un modello murino di aterosclerosi. Ci siamo perciò proposti di indagare se anche le cellule endoteliali e muscolari del macrocircolo umano esprimessero i recettori melanocortinici e quali fossero le loro funzioni.

Metodi: l'espressione dei componenti del MCS nelle cellule endoteliali (HAoEC) e muscolari (HAoSMC) umane aortiche è stata analizzata tramite real-time PCR (qRT-PCR), western blot (WB), immunocitochimica (ICC) e immunoistochimica (IHC). La concentrazione intracellulare di adenosina monofosfato ciclica (cAMP) è stata misurata con un saggio ELISA, mentre la concentrazione intracellulare di calcio nelle EC è stata analizzata tramite indicatori fluorescenti di calcio. La migrazione direzionale delle EC e delle SMC è stata indagata con un saggio che valuta la chiusura del fronte cellulare; la trasmigrazione delle SMC verso un gradiete di chemoattraente (chemiotassi) è stata analizzata con un sistema transwell. Abbiamo studiato l'effetto sulla proliferazione misurando un intercalante fluorescente del DNA sintetizzato *de novo*. In seguito al trattamento con α-MSH, sono stati misurati i profili di espressione genica nel tempo nelle EC e SMC aortiche migranti, usando rispettivamente microarray e sequenziamento dell'RNA. I cambiamenti fenotipici delle cellule muscolari e i biomarcatori associati sono stati analizzati tramite ICC e qRT-PCR.

Risultati: Abbiamo mostrato che le cellule endoteliali e muscolari umane aortiche esprimono recettori melanocortinici funzionalmente attivi. Le EC esprimono MC1R ma non altri componenti del sistema melanocortinico, nè il precursore POMC. L'attivazione del recettore, tramite il ligando naturale α-MSH, accelera la migrazione delle EC in un saggio direzionale *in vitro*. Questo evento è associato ad un aumento intracellulare di calcio e all' inibizione dell'aumento di cAMP. L'analisi dell'espressione genica nel tempo sulle EC migranti ha permesso di identificare una co-regolazione dinamica di geni coinvolti nell'interazione matrice-recettore, nel traffico di vescicole e nella sensibilià ai metalli, tutti processi che hanno una rinomata importanza nella migrazione delle EC.

Le SMC esprimono MC1R e MC4R, ma non POMC. *In vitro,* il trattamento con α-MSH favorisce il differenziamento delle HAoSMC da un fenotipo sintetizzante ad un fenotipo contrattile. Il trattamento promuove un significativo allungamento morfologico delle cellule (picco d'effetto tra 6-12 ore) e un aumento dell'espressione genica e proteica di α-smooth-muscle actin e smooth-muscle-22-α, marcatori del fenotipo contrattile. L'attivazione di MCR rallenta la migrazione delle SMC in un saggio di migrazione direzionale wound-healing (effetto massimo tra 9-12h) e in un saggio chemotattico. Abbiamo mostrato che MC1R è direttamente coinvolto nel processo di migrazione. Inibendo questo recettore con un antagonista specifico (MSG-606) si ristabilisce un normale livello di migrazione, cosa che non accade inibendo invece MC4R. Inoltre, l'attivazione di MCR diminuisce la proliferazione delle SMC. Abbiamo infine indagato le vie di segnalazione attivate a valle di MC1R nelle SMC migranti analizzando il trascrittoma e proteine specifiche. In particolare abbiamo riscontrato una modulazione negativa della via di segnalazione p38MAPK/HSF1 e una significativa riduzione della fosforilazione di p38MAPK in seguito al trattamento con α-MSH. Tale via di segnalazione potrebbe essere il meccanismo di MC1R per il controllo della migrazione delle SMC.

Conclusioni: questi risultati mostrano una nuova funzione dei recettori melanocortinici periferici, *i.e.* la regolazione della motilità delle EC e delle SMC e il mantenimento dell'omeostasi vascolare. Inoltre, questo è il primo studio che si propone di svelare il possibile coinvolgimento dei MCR, espressi a livello del macrocircolo, nella fisiopatologia del rimodellamento vascolare. Infatti, i risultati di questo studio suggeriscono i MCR come nuovi e promettenti target terapeutici per la prevenzione e il trattamento del rimodellamento vascolare patologico mediante il controllo dell'infiammazione vasale, della disfunzione endoteliale e dello switch fenotipico delle SMC.

INTRODUCTION

The term vascular remodeling defines the structural changes in blood vessel geometry that occur in response to long-term physiologic alterations in blood flow or in response to vessel wall injury brought about by trauma or underlying cardiovascular diseases (CVDs) (Gibbons and Dzau 1994; Herity, Ward et al. 1999). The most common CVDs: hypertension, atherosclerosis, and post-angioplasty restenosis arise from maladaptive vascular remodeling characterized by inflammation, endothelial dysfunction, vascular smooth muscle cell alterations, and neointima formation. An estimated 17 million people die every year of CVDs, the primary cause of world death. The goal for health improvement is to identify new ways to treat CVDs. As a contribution to this objective, the specific interest of this thesis was to investigate the cardiovascular actions and mechanisms of melanocortins to identify and characterize new treatment targets and modalities to control maladaptive vascular remodeling.

1. **The Melanocortin system**

The melanocortin system is an ancient hormonal modulatory system that appeared very early in evolution (Catania, Gatti et al. 2004). It is composed of peptides derived by the precursor prohormone proopiomelanocortin (POMC), five G-protein coupled receptors (GPCRs), specific antagonists and ancillary proteins.

POMC is mainly expressed in the corticotrophs and melanotrophs of the anterior and intermediate lobes of the pituitary gland. In addition, it is found in other parts of the central nervous system (CNS), including the arcuate nucleus of the hypothalamus and nucleus of the solitary tract, and in peripheral tissues such as the skin, pancreas, gastrointestinal tract, kidney, and liver (Smith and Funder 1988). The POMC gene encodes a protein precursor that generates bioactive peptides, including adrenocorticotropic hormone (ACTH), α-, β-, and γ-melanocyte stimulating hormone (α-MSH, β-MSH, and γ-MSH) and β-endorphin, via several post-translational modification processes (Takahashi and Mizusawa 2013). Specific enzymes, the pro-hormone convertases, PC1/3, and PC2, make post-translational processing of POMC. In the anterior pituitary, POMC is cleaved by the PC1/3 into ACTH, β-LPH, and an N-terminal fragment. In the intermediate lobe and in the hypothalamus, POMC is more extensively processed by PC2 to produce α-MSH and CLIP from ACTH, and γ-MSH from the N-terminal fragment (Takahashi and Mizusawa 2013; Wolf Horrell, Boulanger et al. 2016) (**Figure 1**). Once produced, α-MSH undergoes a maturation process: α-MSH is N-acetylated by an unidentified

N-acetyltransferase. This acetylation stabilizes α-MSH against proteolytic degradation and prolongs its biological activity. Nevertheless, the half-life of α-MSH is very short since it is still rapidly metabolized (Catania, Gatti et al. 2004).

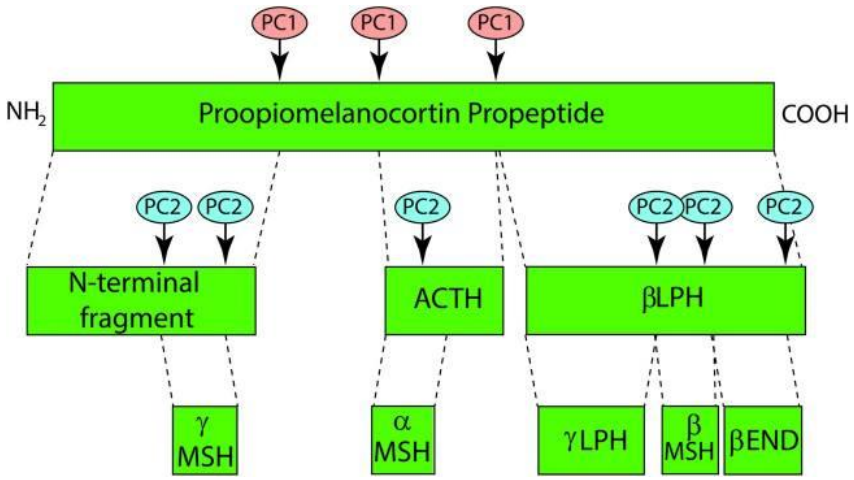


Figure 1. POMC processing (Wolf Horrell, Boulanger et al. 2016).

According to the current understanding, the pituitary gland releases POMC-derived peptides into circulation. The melanocortin peptides reach the peripheral tissues and function in an autocrine or paracrine manner on local melanocortin receptors (Cawley, Li et al. 2016).

The melanocortin system also has two endogenous antagonists, Agouti signaling protein (ASIP) and Agouti-related protein (AgRP); no inhibitory proteins have been identified for any of the seven-transmembrane GPCR family. ASIP and AgRP are paracrine-signaling molecules, which bind to the melanocortin receptors (MCRs) with different affinities. They act as competitive antagonists and finely regulate receptors activities. Binding of ASIP and α-MSH to MC1R are mutually exclusive (Ollmann, Lamoreux et al. 1998). In addition, ASIP functions as an inverse agonist to decrease basal MC1R signaling (Lu, Willard et al. 1994) working with two major accessory proteins: attractin and mahogunin. Attractin is encoded by the *Atrn* gene, is a large single transmembrane protein with an ectodomain that binds the amino terminus of ASIP while Mahogunin is encoded by the *Mgrn1* gene, and is an intracellular protein. Mutations in these ancillary proteins are associated with variations in pigmentation (He, Eldridge et al. 2003). AgRP is an orexigenic neuropeptide that is synthesized in the arcuate nucleus of the hypothalamus in the brain and acts as an endogenous antagonist of the MC3R and MC4R. Additionally, it has been demonstrated that AgRP is an inverse agonist of MC4R

that suppress basal cyclic adenosine mono-phosphate (cAMP) generation (Wilczynski, Joseph et al. 2005).

2. The Melanocortin receptors (MCRs)

Five different MCRs have been cloned and characterized; all the MCRs have seven trans-membrane domain, are coupled to adenylate cyclase and belong to the superfamily of GPCRs (Gantz, Miwa et al. 1993). The MCRs are the product of small genes, many of which are polymorphic. They show high sequence homologies, ranging from 60% identity between MC4R and MC5R to 38% identity between MC2R and MC4R (Catania, Gatti et al. 2004; Yang 2011). Melanocortin receptors are the smallest GPCRs known, with short amino- and carboxyl-terminal ends and a very small second extracellular loop. Human melanocortin receptors also lack several features found in most GPCRs: one, or two, cysteine residues in the first and second extracellular loops and prolines found in the fourth and fifth transmembrane (TM) domains. The melanocortin receptors contain conserved cysteine residues at a different region of the receptor, including cysteine residues within extracellular loops (ELs) and cysteine residues within TMs, and a cysteine residue in C terminus. Functional studies indicate that cysteines in EL3 may form a disulfide bond which is crucial for receptor function while cysteine residues in TMs of the human melanocortin receptors may have different roles in receptor expression, ligand binding and receptor activation (Yang 2011; Herraiz, Garcia-Borron et al. 2017) (**Figure 2**).

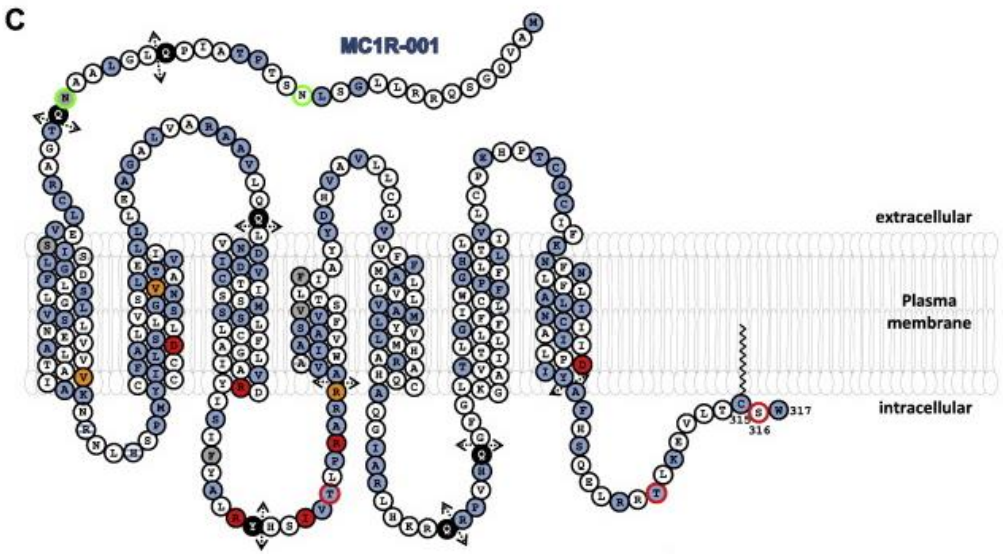


Figure 2. Amino acid sequence, coding sequence polymorphisms and possible arrangement of transmembrane regions in MC1R isoforms. Polymorphic positions for which no reliable association studies are available are

indicated in blue. Positions of R and r variants are shown in red and orange, respectively. Residues shown in gray correspond to indels and black circles with white lettering followed by broken arrows to premature stop codons. Positions, where both an indel and a point mutation have been found, are shown as blue circles hatched in white. (modified from (Herraiz, Garcia-Borron et al. 2017)

MCRs can alternatively couple to the stimulatory G protein (Gs), or other G proteins (Gi/o, and Gq). MCRs associated to adenylyl cyclase in a Gs-mediated stimulatory fashion lead to an increase in cAMP production, activation of protein kinase A (PKA) and cAMP response element-binding (CREB) phosphorylation. However, melanocortin signaling is also associated with other cAMP-independent pathways such as the $Ca^{2+/}$diacylglycerol/inositol trisphosphate (IP_3) pathways (Eves, Haycock et al. 2003; Elliott, Szabo et al. 2004; Catania, Lonati et al. 2010). Pharmacological inhibition of the cAMP pathway using agents such as N6-phenylisopropyl adenosine caused a-MSH induction of an acute intracellular calcium signal. MCR signaling has also been associated with activation of the MAP kinase (Englaro, Rezzonico et al. 1995), Janus kinase/signal transducer and activator of transcription (Buggy 1998), and protein kinases C (PKC) pathways (Kapas, Purbrick et al. 1995). Furthermore, MCRs may be regulated by phosphorylation since they have recognition sites for PKA and C.

Receptors complexity is also increased by the fact that MCRs, as part of the GPCR family, are finely regulated *in loco.* Different responsiveness can occur via altered receptor density (up- or down-regulation), modifications of the receptor such as phosphorylation that diminishes receptor-G protein interaction (uncoupling), and trafficking of receptor away from G protein (sequestration/internalization) that enables either recycling of receptor to a responsive form or facilitates receptor loss by lysosomal degradation (Billington and Penn 2003). Moreover, melanocortin peptides selectively bind to an activate melanocortin receptors with different affinities (Butler, Girardet et al. 2017) controlling various functions, such as energy homeostasis, pigmentation, inflammation (**Figure 3**).

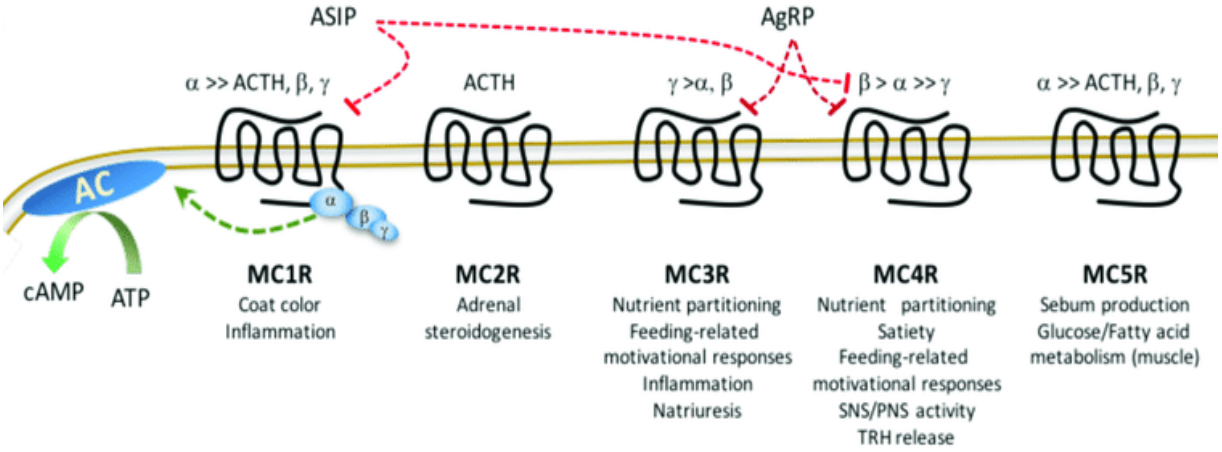


Figure 3. Melanocortin receptors, peptide affinities and mainly functions (Adapted by Butler, Girardet et al. 2017).

2.1. **MC1R**

The MC1R was the first of the melanocortin receptors to be cloned (Mountjoy, Robbins et al. 1992). It was originally cloned from cDNA libraries and detected in melanoma cells. The expression level of the MC1R is relatively low in normal melanocytes but it becomes markedly upregulated upon transformation of melanocytes into malignant melanoma (Loir, Perez Sanchez et al. 1999). MC1R has a high affinity for α-MSH and ACTH, and is also expressed in keratinocyte, fibroblasts, immune cells (neutrophils, monocytes, dendritic cells, B-lymphocytes), glial cells, and endothelial cells (Catania, Gatti et al. 2004).

Like many other GPCRs, oligomerization of MC1R is functionally important to modulate the ligand binding, coupling efficiency, desensitization, and trafficking through the endoplasmic reticulum (Sanchez-Laorden, Sanchez-Mas et al. 2006; Wolf Horrell, Boulanger et al. 2016). Desensitization and internalization also represent major mechanisms whereby receptor function can be regulated (Pitcher, Freedman et al. 1998). MC1R undergoes homologous desensitization following short exposure to its positive agonist, α-MSH, in a PKA-independent and G protein-coupled receptor kinase (GRK) dependent manner. Following agonist stimulation, GRKs phosphorylate GPCRs resulting in receptor decoupling from the G protein and subsequent internalization (Wolf Horrell, Boulanger et al. 2016).

Among the five MCRs identified, MC1R has the highest affinity for α-MSH. MC1R activation controls melanogenesis and has a crucial role in determining the balance between phaeomelanin (yellow) over eumelanin (brown/black) pigment production (Cone 2006). In

humans, recessive mutations within MC1R that render the receptor unresponsive to melanocortin stimulation result in a red hair phenotype (Rees 2003). MC1R has a crucial role also as a mediator of immune responses. MC1R stimulation elicits anti-inflammatory activities; reduce the production of pro-inflammatory cytokines while concomitantly increasing the production of anti-inflammatory cytokines (Bohm, Schiller et al. 2006). The MC1R is present also in the central nervous system but only in the periaqueductal gray matter of the midbrain. This central MC1R might have a role in pain modulation (Xia, Wikberg et al. 1995).

In order to study the different receptor physiology and functions, super potent modified peptides selective and /or specific for all the subtypes have been developed. For example, acetylated NDP-MSH, also known as afamelanotide or Melanotan-I, is a synthetic analog of α-MSH developed to increase the short half-life of the physiologic peptide. It is an agonist at melanocortin receptors with Ki values: 0.085, 0.4, 3.8 and 5.1nM for MC1, MC3, MC4, and MC5 receptors respectively. In comparison with α-MSH and the other natural melanotropins, it possesses better resistance to enzymatic breakdown and it was demonstrated to have prolonged and more potent activity (Sawyer, Sanfilippo et al. 1980). Among the specific agonists of MC1R, one of the most potent and selective developed in the last years is BMS-470539. It worked as a full agonist of recombinant human MC1R with an EC_{50} of 16.8 nM and an intrinsic activity of 88% in a cAMP accumulation assay. It was shown that BMS-470539 exhibited anti-inflammatory properties following ischemia-reperfusion in the vasculature and inhibited leukocyte trafficking (Kang, McIntyre et al. 2006). Among the specific antagonist, 153N-6 and MSG 606 are the most used. The 153N-6 peptide is a putative alpha-MSH antagonist at melanocortin receptor subtypes. The 153N-6 peptide can competitively inhibit NDP-MSH binding from all the receptor subtypes. The relative potency order of 153N-6 for inhibiting NDP-MSH binding was MC1R (Ki 955 +/- 35.7nM) = MC4R (Ki 1151 +/- 106nM) > MC3R (Ki 3229 +/- 637nM) > MC5R (Ki 6286 +/- 462nM) (Chhajlani 1996). MSG 606 is a potent human MC1R antagonist (IC_{50} = 17nM) and it is a partial agonist at human MC3R and MC5R (EC_{50}values are 59 and 1300nM, respectively). It was shown that MSG 606 exhibited binding affinity for A375 melanoma cells *in vitro* (Cai, Stankova et al. 2013) and reversed morphine-induced hyperalgesia in female mice, with no effect in male mice (Arout, Caldwell et al. 2015).

2.2. **MC2R**

The MC2R, unlike the other MCRs, bind only ACTH. It is expressed in the adrenal cortex zona reticularis and zona fasciculata, where it mediates the effects of ACTH on steroidogenesis and

steroid secretion. In addition to the adrenal gland, MC2R seems to be present in the white adipose tissue in mice but not in humans (Boston and Cone 1996), indicating that there are species differences in the expression profiles of the MC2R.

2.3. **MC3R**

The MC3R is the only MCR that responds to stimulation with γ-MSH at physiological concentrations. Thus, γ-MSH seems to be the natural ligand. MC3R is expressed in the central nervous system as well as in multiple tissues in the periphery, including gut-intestinal tract, placenta, heart, kidney and immune cells (Catania, Gatti et al. 2004).

MC3R appears to participate in the modulation of autonomic functions, feeding, and inflammation (Abdel-Malek 2001; Getting, Allcock et al. 2001). α-MSH activation of MC3R and MC4R located in the medullary dorsal-vagal complex mediated hypotension and bradycardia (Li, Varga et al. 1996). MC3R-deficient mice showed an increased fat mass, reduced lean mass, and a higher ratio of weight gain to food intake suggesting the participation of MC3R in energy homeostasis (Chen, Marsh et al. 2000). MC3R activation mediates the protective influences of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias in rats (Guarini, Schioth et al. 2002). Furthermore, activation of MC3R has clear anti-inflammatory influences (Getting and Perretti 2000; Getting, Christian et al. 2002).

2.4. **MC4R**

The human MC4R was the second neural MCR to be cloned. Alignment of MC4R with other MCRs showed that it has the highest homology with the MC3R, with 58% identity and 76% similarity. The order of potency for activation of MC4R is α-MSH > ACTH > β-MSH >> γ-MSH. MC4R is mainly expressed in the CNS where it has a very wide distribution, including the cortex, thalamus, hypothalamus, brain stem and spinal cord (Mountjoy, Mortrud et al. 1994). Distribution of MC4R is consistent with its involvement in autonomic and neuroendocrine functions (Catania, Gatti et al. 2004). In addition, the MC4R mRNA is also expressed in peripheral tissues during the fetal period, including the developing heart (E14), lung (E16), muscles involved in respiration such as diaphragm and intercostal muscle (E14), as well as other muscles (Mountjoy, Jenny Wu et al. 2003; Tao 2010) . The potential physiological functions of the MC4R in these organs remain to be elucidated.

MC4R regulates food intake and energy homeostasis. Knock out mice for MC4R show maturity-onset obesity, with hyperphagia, hyperinsulinemia, and hyperglycinemia (Tao 2010).

Accordingly, the pharmaceutical industry is looking at MC4R as a target to treat weight disorders. This receptor has been associated with several physiological functions, including sexual function and pain, but the most important role for MC4R is certainly in the control of energy balance and cardiovascular functions.

To study the receptor functions specifically, selective agonists and antagonists have been developed. JKC 363 is a potent and selective MC4R antagonist (IC_{50} values are 0.5 and 44.9nM at MC4R and MC3R respectively) that contrapose the effects of α-MSH. It suppresses thyrotropin-releasing hormone release (Kim, Small et al. 2002), attenuates food intake (Verty, McFarlane et al. 2004) and reduces formalin-induced pain *in vivo* (Bellasio, Nicolussi et al. 2003). PG 931, instead, is a potent and selective MC4R agonist (IC_{50} values are 0.58 and 55nM for human MC4R and MC3R respectively). It was shown that it produced a rapid and dose-dependent restoration of cardiovascular and respiratory function in hemorrhage-shocked rats (Grieco, Balse-Srinivasan et al. 2003; Giuliani, Mioni et al. 2007).

2.5. **MC5R**

MC5R is similar to the MC1R and MC4R in its capacity to recognize α-MSH and ACTH but not γ-MSH (α-MSH > ACTH >> γ-MSH)(Catania, Gatti et al. 2004). The MC5R was the last of the MCR gene family to be cloned by homology screening from genomic DNA in man (Chhajlani, Muceniece et al. 1993), mouse (Labbe, Desarnaud et al. 1994), and rat (Griffon, Mignon et al. 1994).

MC5R is a relatively ubiquitous receptor in peripheral tissues but it is widely expressed in the secretory epithelia of many exocrine glands such as the adrenal, lacrimal and sebaceous glands (Chhajlani 1996; Catania, Gatti et al. 2004). This kind of distribution indicates that MC5R is involved in regulating the synthesis and secretion of exocrine gland products (Chen, Kelly et al. 1997). However, data also indicate immunomodulatory functions in B and T lymphocytes and in mast cell lines (Buggy 1998; Taylor and Lee 2011).

3. **Vascular structure and homeostasis**

The vascular system of the human body is comprised of arteries, capillaries, and veins that contribute to cellular function, absorption of essential nutrients, and removal of cellular and metabolic waste products maintaining the cell homeostasis. The arteries and veins structure (**Figure 4**) is similar and composed of three layers. Each layer exhibits specific histologic,

biochemical and functional characteristics to maintain vascular homeostasis and to regulate the vascular response to stress or injury. The Tunica intima is composed of epithelial and connective tissue. The specialized monolayer of epithelium (the endothelium), is responsible for creating an interface between the blood and tissues and plays a role in blood vessel dilation and constriction. Next to the endothelium is the basement membrane or basal lamina that binds the endothelium to the connective tissue. The basement membrane provides strength while maintaining flexibility and it is permeable. The middle layer, Tunica media is the thickest layer in arteries and consists of smooth muscle cells arranged in circular sheets supported by connective tissue, primarily made up of elastic fiber. The outer tunica, the tunica *externa* or adventitia, is a substantial sheath of connective tissue composed primarily of collagenous fibers that exert protective functions.

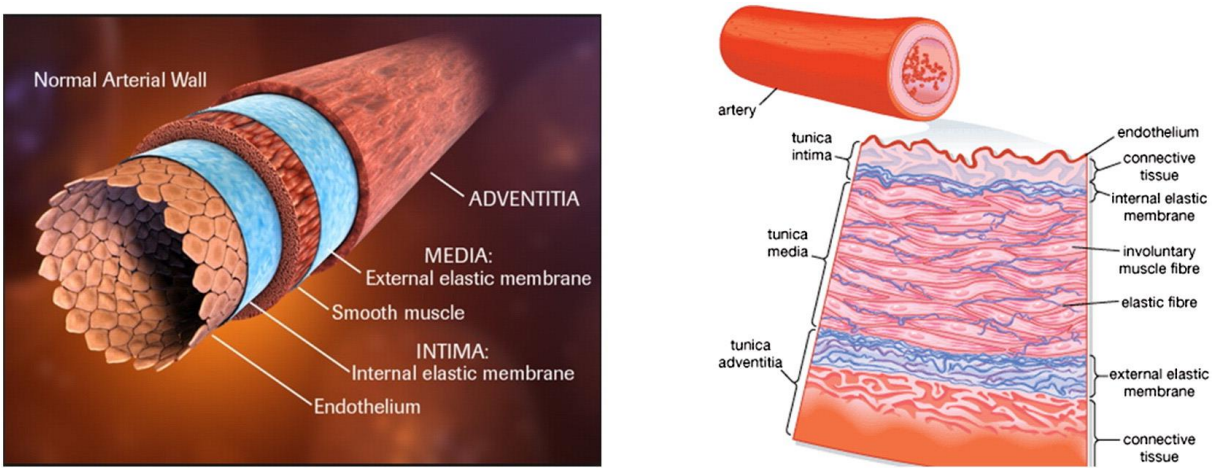


Figure 4. Vascular structure (adapted from Fortier A. et al.2014)

3.1. **Endothelial cell**

The endothelium is the main regulator of vascular wall homeostasis. Physiologically, endothelial cells maintain a relaxed vascular tone and low levels of oxidative stress by releasing mediators such as nitric oxide, prostacyclin and endothelin, and controlling local angiotensin-II activity. The endothelium also actively regulates vascular permeability to plasma constituents, platelet and leukocyte adhesion and aggregation, and thrombosis (Sitia, Tomasoni et al. 2010).

However, a number of conditions altered the endothelial regulation of blood vessel function. In response to a variety of noxious stimuli, the endothelium undergoes phenotypical modulation to a non-adaptive state known as “endothelial dysfunction”, which is characterized by the loss or dysregulation of the homeostatic mechanisms that operate in

healthy endothelial cells (Pepine 1998; Della Rocca and Pepine 2010). This pathophysiological condition is associated with the increased expression of adhesion molecules, an increased synthesis of pro-inflammatory and pro-thrombotic factors, increased oxidative stress, and the abnormal modulation of vascular tone, all of which may lead to functional manifestations including impaired endothelium-dependent vasodilation. Depending on the pathology, vascular bed and the presence of other risk factors such as diabetes and obesity, the mechanisms underlying endothelial dysfunction may be multifactorial and considerably different from each other (Sitia, Tomasoni et al. 2010). Current evidence suggests that endothelial dysfunction occurs early in the process of atherogenesis, and contributes to the formation, progression, and complications of atherosclerotic plaque (**Figure 6**).

In hypertensive vessels and in the first steps of atherosclerosis progression, the expression of eNOS enzyme by the endothelium is paradoxically increased, indicating that the ability of eNOS to produce NO has become compromised and/or the bioavailability of NO has declined (Harrison 1997; Gimbrone and Garcia-Cardena 2016). Reduced NO bioavailability can arise from attenuated production but also from the increased breakdown of NO by reactive oxygen species. Thus, oxidative stress is one of the main mechanisms contributing to endothelial dysfunction (Touyz and Briones 2011). Finally, in healthy arteries, the endothelium serves as non-adhesive and anti-thrombotic surface thereby providing vascular protection. Since endothelium-derived vasodilator substances exert anti-thrombotic and anti-proliferative actions and contribute significantly to the protective role of endothelium in vascular homeostasis, an impairment of endothelium-dependent vasodilatation is often accompanied by dysregulation of endothelial-blood cell-interaction, which may lead to inflammation, vascular lesions, and thrombosis.

When the balance between EC NO production and oxidant stress is disrupted, ECs become dysfunctional. Vascular SMC relaxation is impaired and its phenotype switches to a synthetic form that promotes growth, proinflammatory responses, and thrombotic activities. Eventually, these functional defects result in structural damage to the arterial wall with SMC migration and proliferation in the media and sub-intima, creating favorable conditions for platelet and leukocyte activation and adhesion as well as cytokines that increase permeability to oxidized lipoproteins and inflammation mediators. Hence, endothelial dysfunction leads to adaptive vascular remodeling and it is pivotal to atherogenesis. It is present at the earliest

stages as well as later stages of the arterial disease, contributing to clinical outcomes related to tissue damage (ischemia, infarction, and organ failure) (**Figure 6**).

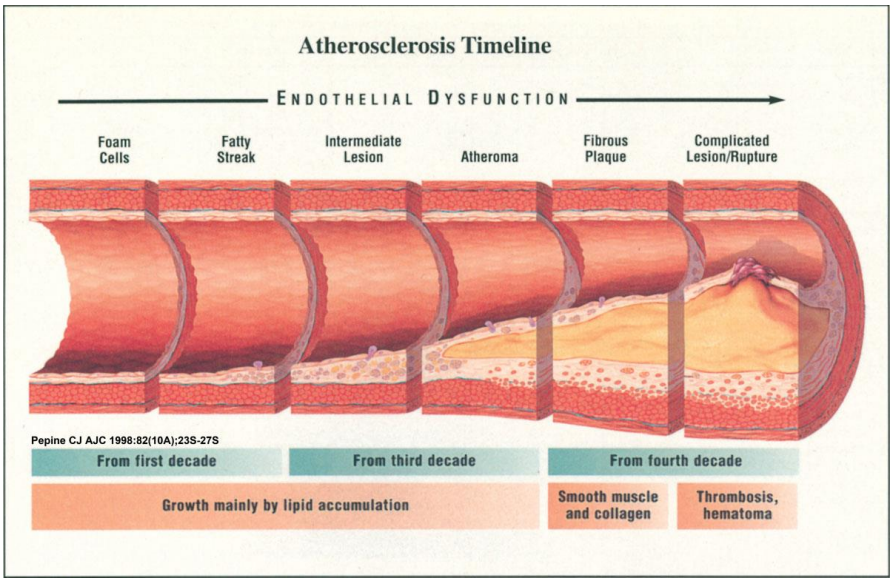


Figure 6. Atherosclerosis timeline, showing the underlying role of endothelial dysfunction in the progression of atherosclerosis from the initial lesion to the complicated lesion (Della Rocca and Pepine 2010).

3.1.1. **Endothelial cell migration**

Endothelial cells migrate during vasculogenesis and angiogenesis, but also in a damaged vessel to restore vessel integrity. Artery endothelial dysfunction and/or injury are linked to the pathogenesis of atherosclerosis, thrombosis, or surgery procedure complications (Otsuka, Finn et al. 2012). An essential biological process involved in endothelial healing upon vascular injury is EC migration. When a blood vessel is damaged, the restoration of endothelium and vessel integrity is achieved through migration of healthy ECs to the site of the lesion and subsequent proliferation. Hence, EC migration has a key role, besides angiogenesis, in vascular repair and tissue regeneration (Michaelis 2014; Hasan and Siekmann 2015). Understanding and accelerating the mechanisms of endothelial wound healing is of fundamental interest for biotechnology and of significant medical utility in repairing pathologic changes to the vasculature induced by invasive medical intervention.

Endothelial cell migration is a mechanically integrated molecular process that involves changes in cell adhesion, signal transduction, and cytoskeletal organization. It involves three major mechanisms, namely chemotaxis, the directional migration toward a gradient of soluble chemoattractants; haptotaxis, the directional migration toward a gradient of immobilized ligands; and mechanotaxis, the directional migration generated by mechanical forces

(Michaelis 2014). Chemotaxis of endothelial cells is driven by growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, whereas haptotaxis is associated with increased endothelial cell migration activated in response to integrins binding to extracellular matrix component. Mechanotaxis is because endothelial cells are constantly in contact with shear stress, which contributes to activate the migratory pathway.

The migratory pathway identified in endothelial cells comprise migration initiation, filopodia and lamellipodia formation and cell polarization (**Figure 7**).

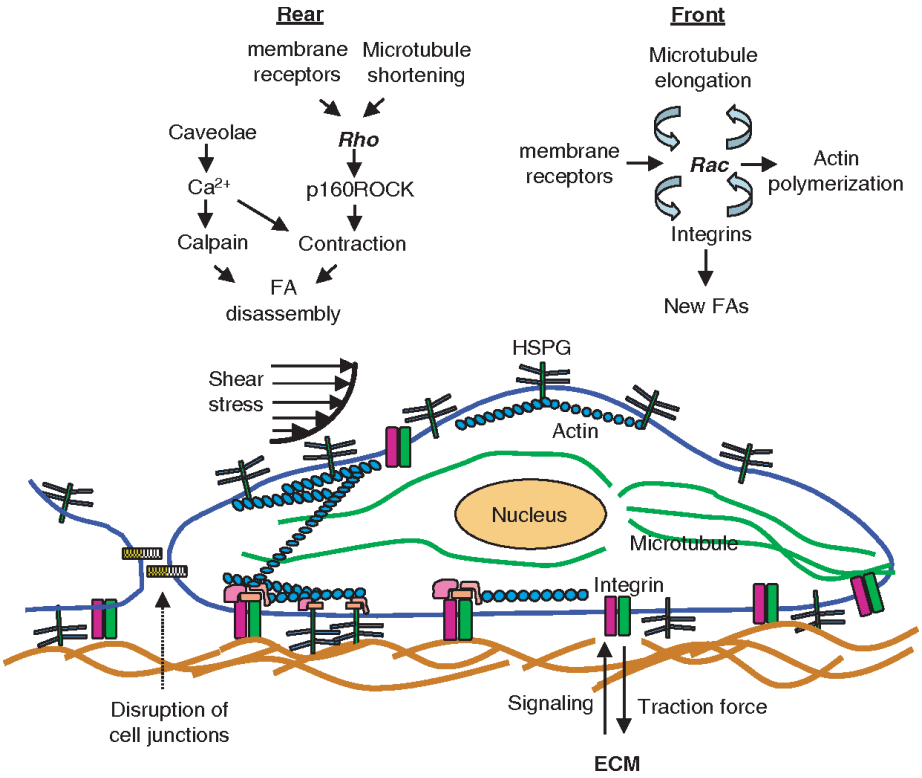


Figure 7. A model of the polarized activation of signaling molecules during EC migration (Li, Huang et al. 2005).

Two important genetic pathways influencing these processes are the VEGF and the Notch signaling pathways that control angiogenesis (Michaelis 2014). The Serum response factor is an evolutionarily conserved transcription factor known to be important for filopodia formation, needed for cell movements (Franco, Blanc et al. 2013). Moreover, MAP4K4 seems to be essential in the retraction of the plasma membrane at the rear end that contrapose the extension of protrusions in the direction of cell migration (Vitorino, Yeung et al. 2015). Rac1, Cdc42 and RhoA control EC migration via changes in cytoskeletal arrangement regulating the formation of lamellipodia, filopodia and stress fibers (Li, Huang et al. 2005). Among these, the Calcium signaling pathway has essential roles in EC migration. Ca^{2+} is regulated spatially and temporally in migrating cells, driving polarization, protrusion, retraction, and adhesion at the

right place and right time. Cells create local Ca^{2+} pulses near the leading edge, maintain cytosolic Ca^{2+}gradient from back to front, and restore Ca^{2+}depletion for persistent cell motility (Tsai, Kuo et al. 2015).

3.2. **Vascular Smooth Muscle Cell (SMC)**

Vascular smooth muscle cells are specialized cells, mainly located in the tunica media of blood vessels, responsible for contraction and relaxation to maintain blood pressure and blood flow distribution. SMCs within the adult blood vessel proliferate at an extremely low rate and exhibit a very low synthetic activity.

SMCs express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for the contractile function that is unique compared with other cell types and other muscle lineages including the skeletal muscle and cardiac muscle (Owens, Kumar et al. 2004). SMCs contraction occurs by Ca^{2+} /calmodulin phosphorylation of myosin light-chain kinases that phosphorylates myosin light chain. Phosphorylated myosin light chain form cross-bridges with actin. Contraction is antagonized by myosin light-chain phosphatase, the activity of which is negatively regulated by Rho-kinase, PKC-dependent 17kDa C-protein-potentiated inhibitor of protein phosphatase, and positively regulated by endothelial cell-derived nitric oxide through soluble guanylate cyclase-cGMP-PKG. In vascular SMCs, intracellular Ca^{2+} concentrations may be increased, to induce contraction, by influx from outside the cell through voltage-gated calcium channels that activate upon cellular depolarization or from the sarcoplasmic reticulum by ryanodine or IP_3 receptor channels. Either excitation-contraction coupling *(i.e.*, action potentials) or pharmaco-mechanical coupling induce contraction/relaxation in SMCs (Misono, Philo et al. 2011). SMC responses are driven through the activation of membrane receptors, among which GPCRs are the most common. GPCRs are activated by endogenous agonists including hormones and neurotransmitters (prostaglandins, Angiotensin II, Adrenalin). Several GPCRs, such as the angiotensin II AT_1 receptor, the endothelin-1 ET_A receptor, and the α1 -adrenoceptor mainly use the $G_{q/11}$-PLC signaling pathway to stimulate vasoconstriction. Other receptors, such as the α2-adrenoceptor, mediate contraction via $G_{i/o}$ proteins. Relaxation of vascular SMCs is mainly mediated by G_s protein-coupled receptors, which reduce the Ca^{2+} sensitivity (Maguire and Davenport, 2005).

Unlike either skeletal or cardiac muscle cells that are terminally differentiated, SMCs within adult animals retain remarkable plasticity and can undergo rather profound and reversible

changes in phenotype in response to changes in the local environment (Owens, Kumar et al. 2004) (**Figure 8**).

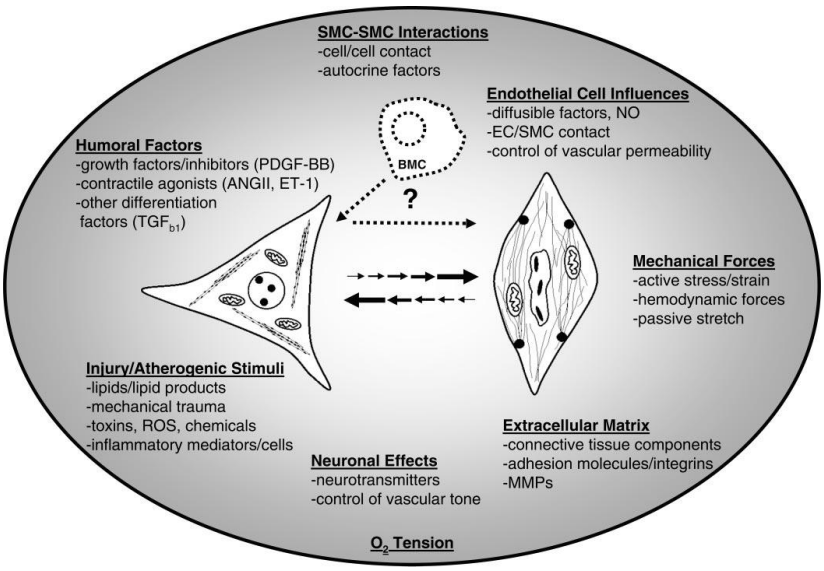


Figure 8. The SMC phenotypic switch in response to the local environment (Owens, Kumar et al. 2004).

To perform a diversity of functions, SMC phenotype spans a continuum from quiescent and contractile to proliferative and synthetic (Owens, Kumar et al. 2004; Beamish, He et al. 2010; Milewicz, Kwartler et al. 2010). The diversity between phenotypes becomes apparent in morphology, expression levels of SMC marker genes, proliferative potential and migration properties (**Figure 9**). Contractile SMCs have a fully functional contractile apparatus that responds to small molecule signals such as acetylcholine and norepinephrine. Contractile SMCs are characterized by an ultrastructure composed of myofilaments and minimal rough endoplasmic reticulum, Golgi, or free ribosomes. In culture, these cells possess a dense fusiform morphology. Synthetic SMCs have a fibroblast-like aspect, contain minimal contractile proteins and secrete ECM. The ultrastructure of these cells shows a cytoplasm devoid of contractile bundles with extensive rough endoplasmic reticulum, Golgi, and ribosomes. In culture, these cells adopt a spread, rhomboid shape, and then begin to grow over one another in a ‘‘hill-and-valley’’ morphology (Beamish, He et al. 2010; Milewicz, Kwartler et al. 2010). The synthetic phenotype is also correlated with SMC proliferation that increases from 3%–5% to 40%–60% during primary culture and pathologies such as intimal hyperplasia. Finally, synthetic SMCs show higher migratory activity than contractile SMCs (Rensen, Doevendans et al. 2007).

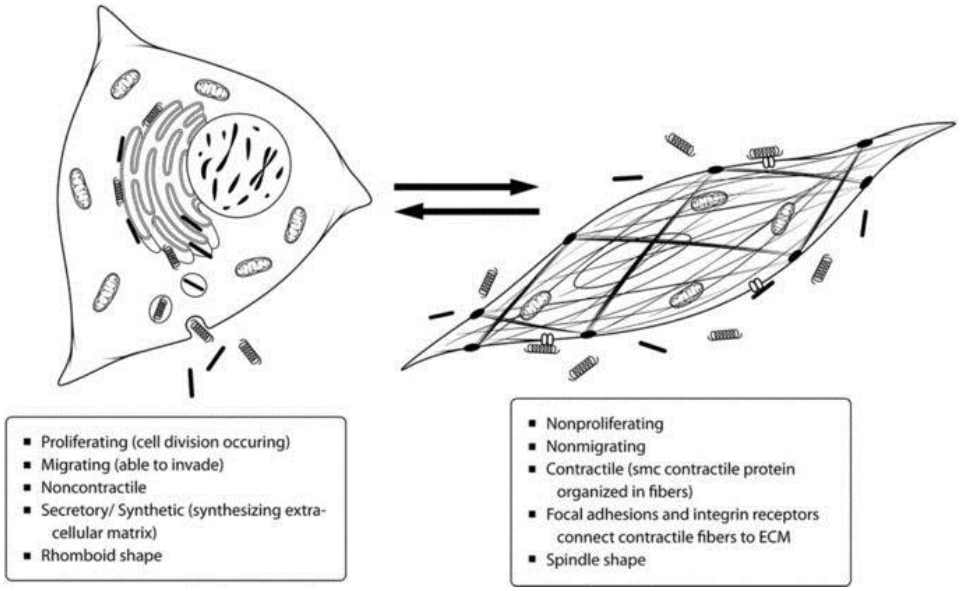


Figure 9. Main differences in SMCs phenotypes. (Milewicz, Kwartler et al. 2010)

The expression patterns of a wide range of protein markers have been characterized to describe the phenotypic state of SMCs (Table 1). Contractile SMCs, which predominate in normal vessels, exhibit a mature contractile apparatus including α-SMA, smooth muscle myosin heavy chains (SM-MHC), MHY11, Calponin, SM22α, and Smoothelin. There also are several markers of synthetic SMC phenotype, such as Caldesmon, MHY10 (Smemb) and Vimentin, but these have been utilized less widely in the literature and generally, these markers have fewer SMC specificity. Markers that are upregulated in the synthetic phenotype are rare. Instead, the disappearance of proteins associated with the contractile phenotype is generally taken as characteristic of the synthetic phenotype (Rensen, Doevendans et al. 2007; Beamish, He et al. 2010).

SMC plasticity is essential during vascular development when the SMCs play a key role in the morphogenesis of the blood vessel and exhibits high rates of proliferation, migration, and production of extracellular matrix components. Similarly, in response to vascular injury, the SMC dramatically increases its rate of cell proliferation, migration, and synthetic capacity and plays a critical role in vascular repair. However, SMC high plasticity could bring to adverse phenotypic switching and acquisition of characteristics that can contribute to the development and/or progression of vascular disease. Indeed, there is strong evidence that phenotypic switching of the SMC plays a major role in a number of human diseases including atherosclerosis, cancer, and hypertension (Bennett, Sinha et al. 2016). Extensively

proliferating SMCs play a pivotal role in restenosis that occurs after vascular procedures such as endarterectomy and percutaneous coronary intervention.

Table 1. Characteristics of widely used SMC marker proteins.

Marker protein	SMC specificity	Phenotype specificity	Subcellular localisation	Onset of expression	Function
α-smooth muscle actin	Embryo - Adult +/-	c>s	Contractile filaments	E9 heart/ somites/SMCs	Contraction
Smooth muscle-myosin heavy chain	Embryo + Adult +	SM1 c>s SM2 c	Contractile filaments	E10.5 SMCs	Contraction
SM22α	Embryo - Adult +	c>s	Actin-associated	E8 heart/ E9.5 SMCs	Regulation contraction
SM-calponin	Embryo - Adult +	c>s	Actin-associated/ cytoskeleton	E8.5 heart E13.5 SMCs	Regulation contraction/ signal transduction
H-caldesmon	Embryo + Adult +	c	Actin-associated	WK 10 human	Regulation contraction
Smoothelin	Embryo - Adult +	c	Actin-associated	E13	Regulation contraction
Telokin	Embryo+/? Adult +	c>s	Cytoplasmic/ membrane	E11.5 gut	Regulation contraction
Meta-vinculin	Embryo - Adult -	c>s	Cytoskeleton	WK 24 human	Anchoring cell-ECM
Desmin	Embryo - Adult -	c>s	Cytoskeleton	E9.5 myotome	Structural mechanical integrity
CRBP-1	Embryo - Adult -	s>c	Cytoplasm	E10	Retinoid transport and metabolism
Smemb	Embryo - Adult -	s>c	Contractile filaments	ND	Contraction

Compiled from Owens et al. and Miano.[1,5]
c=contractile, s=synthetic, E=mouse embryonic day, WK 24=week 24 of human development, ND=not determined.

Table 1. Characteristics of widely used SMC marker proteins (Rensen, Doevendans et al. 2007)

3.2.1. **Smooth muscle cell proliferation and migration**

During vascular development, SMC migration is guided by chemotactic stimuli, such as platelet-derived growth factor (PDGF), in order to assure the correct vessels formation. Normal vessel formation occurs by formation of an endothelial cell tube followed by migration of pericytes or smooth muscle progenitor cells that differentiate to smooth muscles of the tunica media. In physiologic conditions, the vascular SMCs are in a non-proliferating and non-migrating state, since there are not environmental pro-proliferating and migrating factors; moreover, focal adhesions and junctions between cells and extracellular matrix keep cells in place. Smooth muscle cell migration occurs not only during vascular development, but also in response to vascular injury, and during atherogenesis. Vascular injury occurs clinically after angioplasty, vascular stent implantation, or organ transplantation. In animal models of vascular injury, intimal and medial thickening is thought to be attributable to SMC proliferation as well as migration from media to intima (Zahradka, Harding et al. 2004; Gerthoffer 2007). In response to an inflammatory micro-environment, such as a vascular

lesion, vascular SMC proliferation and migration is induced to repair the injury; however if the stimulus is persistent, excessive ECM deposition lead to vessel thickening. In atherosclerosis, the vascular SMC contributes to vessel wall inflammation and lipoprotein retention, as well as to the formation of the fibrous cap that provides stability to the plaque.

It has been observed that vascular SMC remodeling *in vivo* and *in vitro* begins with an extracellular stimulus that activates receptors located on the cell surface. These, in turn, transduce the external signal to several pathways, leading to a series of coordinated remodeling events that trigger cell migration, proliferation or both. Signaling pathways that are activated when exposed to mitogens, growth factors or peptides often trigger pathways that stimulate both proliferation and migration. The main factor that modulates proliferation is PDGF-BB that it is also a well-known pro-migratory chemotactic factor. Pro-migratory stimuli activate signal transduction cascades that trigger remodeling of the cytoskeleton, change the adhesiveness of the cell to the matrix, and activate motor protein (Gerthoffer 2007).

The p38 MAPK cascade it is one of the main pathway implicated in SMC migration. A signaling cascade comprising MAPK kinase 3, p38 MAPKs, MAPK activated protein kinase 2 (MK2), and heat shock protein 27 (HSP27) is necessary for migration of both smooth muscle cells and endothelial cells. The p38 MAPK cascade is activated by numerous pro-migratory stimuli, including PDGF, angiotensin II, S1P, and thrombin. Chemical inhibitors of p38 MAPK block vascular remodeling in a rabbit injury model, although, interestingly, p38 MAPK inhibitions did not stop the migration of cultured cells. Inhibition of p38 MAPK also alters the reorientation of cultured SMC in response to stretch suggesting p38 MAPK and its targets are early sensors of both the chemical and mechanical milieu of the vessel wall. The effect of p38 MAPK inhibitors in vascular remodeling is likely to be a combination of effects on cell migration via inhibiting phosphorylation of HSP27 and actin remodeling, as well as effects on transcription and translation of proinflammatory mediators and proteins regulating the cell cycle (Wang, Castresana et al. 2004).

The p38 MAPK/MK2/HSP27 pathway in smooth muscle is associated with the control of actin filament polymerization and subsequently, cell migration (Salinthone, Tyagi et al. 2008). Hedges and colleagues in 1999 showed that activation of p38 MAPK leading to HSP27 phosphorylation is necessary for migration of tracheal smooth muscle cells (Hedges, Dechert et al. 1999). Studies in airway smooth muscle showed the p38 MAPK signaling pathway mediates phosphorylation of HSP27 in response to serum, growth factors and cytokines (Hedges, Yamboliev et al. 1998). Blocking p38 MAPK activity with SB203580 inhibits activation of MK2, prevents phosphorylation of HSP27 and blocks smooth muscle cell migration (Hedges, Dechert et al. 1999). These studies established that activation of the p38 MAPK pathway by growth factors and pro-inflammatory cytokines promotes cell migration and that phosphorylation of HSP27 is necessary for migration. Subsequent investigations by other labs confirmed that the p38 MAPK/MK2/HSP27 pathway is necessary for migration of vascular smooth muscle cells (Lee, Lee et al. 2007), neutrophils (Jog, Jala et al. 2007), and fibroblasts (Hirano, Rees et al. 2004).

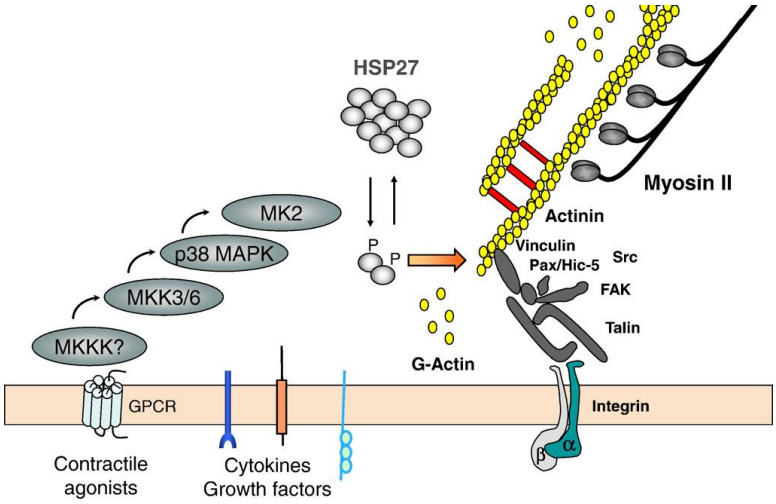


Figure 10. Signaling pathways regulating HSP27 structure and function (Salinthone, Tyagi et al. 2008)

Many, but not all, heat-inducible proteins contain heat shock response elements (HSE) in the 5' untranslated region of the coding sequence (Salinthone, Tyagi et al. 2008). Heat shock response elements are characterized by inverted repeats of nGAAn that are binding sites for transcription factors called heat shock factors (HSF). HSF-1 is the predominant mammalian HSF. Small HSP expression levels are probably controlled in smooth muscles by a combination of transcriptional regulation via heat shock factors, control of mRNA stability and regulation of translation. In many cells expression and phosphorylation of HSF-1 are regulated by stress signals to allow trimerization, binding to the heat shock response elements in the 5'

untranslated regions of HSP genes and transcription of HSP mRNAs. Upstream of HSF-1, p38 MAP kinases probably have important regulatory effects because HSP27 can be induced by vasoactive agents that activate the p38 MAPK cascade including thrombin, vasopressin and sphingosine 1-phosphate (Salinthone, Tyagi et al. 2008). Interestingly the p38 MAPK/MK2 signaling cascade is also the pathway that promotes phosphorylation of HSP27, dissociation of HSP27 multimers and increased F-actin formation in actin cytoskeletal remodeling (**Figure 10**). This suggests dual effects of p38 MAPK on both short-term events promoting HSP27 phosphorylation and in longer-term processes promoting HSP27 expression.

4. **Vascular remodeling**

Vascular remodeling is an active process of structural alteration that involves changes in at least four cellular processes: cell growth, cell death, cell migration, and production or degradation of extracellular matrix and is dependent on a dynamic interaction between locally generated growth factors, vasoactive substances, and hemodynamic stimuli. Remodeling is usually an adaptive process that occurs in response to long-term changes in hemodynamic conditions, but it may contribute to the pathophysiology of vascular diseases and circulatory disorders (Gibbons and Dzau 1994; Herity, Ward et al. 1999). Mechanisms involved in arterial remodeling include fibrosis, hyperplasia of the arterial intima and media, changes in vascular collagen and elastin, endothelial dysfunction, and arterial calcification. Migration and proliferation of vascular smooth muscle cells contribute to thickening of the intima. Differentiation of smooth muscle cells from their contractile to a secretory or osteogenic phenotype may lead to increased vascular tone and promotes extracellular matrix calcification.

There are different types of remodeling. Arterial remodeling can be inward or outward and can be hypertrophic (thickening of the vascular wall), eutrophic (constant wall thickness), or hypotrophic (thinning of the vascular wall) (Mulvany, Baumbach et al. 1996) (**Figure 11**).

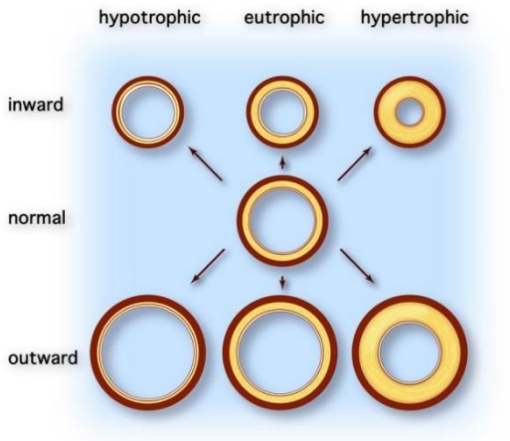


Figure 11. Different types of vascular remodeling (van Varik, Rennenberg et al. 2012)

Hypotrophic remodeling results in a relatively thinner wall and a lower wall-to-lumen ratio. Conversely, hypertrophic remodeling is characterized by thickening of the vascular wall due to cellular hyperplasia and/or hypertrophy or deposition of extracellular matrix material and results in increased wall-to-lumen ratio. When the diameter of the vessel changes but the wall-to-lumen ratio remains the same it is called eutrophic remodeling (van Varik, Rennenberg et al. 2012).

The features of vascular remodeling, intimal hyperplasia, medial hypertrophy, hyperplasia of vascular SMCs, and deposition of ECM, cause thickening of the arterial wall that reduces tensile stress. Vascular adult SMCs synthesize non-elastic collagen causing the stiffening of the vascular wall (Greenwald 2007) that it is increased also by degradation of ECM and the deposition of calcium minerals. In addition to structural changes, endothelial function plays an important role in arterial remodeling. Blood flow and shear stress stimulate endothelial cells to produce nitric oxide, which in turn influences contraction and relaxation of vascular SMCs. Endothelial function decreases with age and endothelial dysfunction is common in many cardiovascular diseases. Moreover, in response to pathological conditions, such as altered shear stress or inflammation, endothelial cells produce cytokines and growth factors that influence the homeostasis of the vascular wall (van Varik, Rennenberg et al. 2012).

SMC proliferation and migration are hallmarks in maladaptive vascular remodeling, cause neointima formation and promote atherosclerosis. Although the benefit/risk of therapeutic inhibition of vascular SMC proliferation in atherosclerosis is unclear, experimental and human evidence prompts the use of anti-proliferative therapy for in-stent restenosis, bypass graft failure and other vascular proliferative disorders (Dzau, Braun-Dullaeus et al. 2002).

Stent implantation has become a widely used procedure to treat vessel occlusion but it is not without complications. Restenosis or the re-narrowing of the arteries are the principal adverse events. In-stent restenosis occurs in 15–20% of patients receiving a bare metal stent for the treatment of simply coronary lesions but may occur in up to 30–60% of patients with complex lesions (Puranik, Dawson et al. 2013). To prevent restenosis and pathological vascular remodeling has been developed drug-eluting stents (DES). The first generation DES are covered with potent anti-proliferative agents such as Sirolimus and Paclitaxel. Second generation stents bring compound more specific, more efficacious and selective anti-proliferative agents that have lower systemic toxicity and do not delay endothelial healing such as Everolimus and Zotarolimus (Byrne, Joner et al. 2015). Furthermore, DES placement is not feasible for alleviating the restenotic response in bypass grafts, transplants, or hemodialysis grafts, and there have been reports of increased risk of late stent thrombosis (Puranik, Dawson et al. 2013). The drug that covers the second-generation stents have anti-proliferative activity and block SMC proliferation, with the secondary adverse event due to the decrease in endothelial proliferation and wound closure. In order to develop better-targeted therapies, with a decreased risk of stent-induced thrombosis, a better understanding of the pathogenesis of endothelial dysfunction and neointimal hyperplasia is required. A better strategy could be the development of new drugs able to control SMC migration and proliferation without affecting EC functions.

5. Involvement of MCRs in inflammation and cardiovascular functions

Preclinical investigations indicate that activation of certain MCR subtypes, primarily MC1R, could be a novel strategy to control inflammatory disorders (Catania, Gatti et al. 2004). The anti-inflammatory influences of α-MSH, the prototypic melanocortin, are exerted through inhibition of inflammatory mediator production and reduction of inflammatory cell migration. Melanocortin peptides exert anti-cytokine and anti-inflammatory effects in blood cells, cells of the immune system, and in other cell types including neural, endothelial, and epithelial cells (Lipton and Catania 1997). Moreover, α-MSH is able to inhibit activation of the nuclear factor-kB (NF-kB) (Manna and Aggarwal 1998), an essential transcription factor expressed in all eukaryotic cells that induces transcription of many molecules involved in the inflammatory

process. It is also known that α-MSH produced within the brain inhibit fever (Lipton, Macaluso et al. 1991), so that peptide could act centrally to modulate inflammation in the periphery. Nevertheless, the peripheral effects of locally expressed melanocortin peptides are poorly described.

Interestingly, a local melanocortin system has been described in endothelial cells of the cutaneous (Hartmeyer, Scholzen et al. 1997) and cerebral (de Angelis, Sahm et al. 1995) microcirculation. Hartmeyer and colleagues showed that human dermal microvascular endothelial cells (HDMEC), as well as transformed human dermal microvascular endothelial cells (HMEC-1), expressed functionally active MC1R. The MC1R expression is upregulated upon stimulation with α-MSH that also increase IL-8 release. This study provides evidence of a local melanocortin system involved in the control of inflammation and leukocyte-endothelial cell interaction (Hartmeyer, Scholzen et al. 1997).

There is increasing evidence demonstrating that the melanocortin system is crucially involved in cardiovascular regulation and atherosclerosis. α-MSH has been shown to modulate blood vessel tone by enhancing nitric oxide-cGMP dependent relaxation responses through endothelial MC1R in mice (Rinne, Nordlund et al. 2013). Moreover, *in vivo* treatment with α-MSH analogs ameliorates endothelial dysfunction in aged and obese mice. Interestingly, treatment with MCR agonists was able to prevent the development of vascular dysfunction and attenuate plaque inflammation in pre-established atherosclerosis in a model of $LDLR^{-/-}ApoB^{100/100}$ mice (Rinne, Silvola et al. 2014). These studies suggested that the anti-inflammatory and vasoactive effects of melanocortins could be therapeutically expanded to chronic inflammatory diseases such as atherosclerosis.

The last studies about melanocortin implication in vascular pathologies showed that deficiency in MC1R signaling predisposes to vascular endothelial dysfunction and increased arterial stiffness in mice and humans (Rinne, Ahola-Olli et al. 2015). Moreover, using a cross model of $Apoe^{-/-}$ mice with recessive yellow ($Mc1r^{e/e}$) mice, carrying dysfunctional MC1R, Rinne and colleagues demonstrated that lacking in MC1R signaling accelerates diet-induced atherosclerosis (Rinne, Kadiri et al. 2018). They also showed that MC1R deficiency was associated with less collagen and smooth muscle cells, enhanced arterial accumulation of monocytes and macrophages and increased necrotic core, all indication of more vulnerable lesions. Finally, MC1R deficiency exacerbates HFD-induced hypercholesterolemia and hepatic lipid accumulation in $Apoe^{-/-}$ mice (Rinne, Kadiri et al. 2018). Accordingly, melanocortin

overexpression limits diet-induced inflammation and atherosclerosis in $LDLR^{-/-}$ mice (Nuutinen, Ailanen et al. 2018). Transgenic mice overexpressing MSH (MSH-OE) had improved glucose tolerance and limited atherosclerotic plaque formation, particularly after Western diet feeding. In terms of aortic vasoreactivity, MSH-OE blunted alpha1-adrenoceptor-mediated vasoconstriction and enhanced relaxation response to acetylcholine, indicating improved endothelial function. In addition, MSH-OE markedly attenuated Western diet-induced upregulation of proinflammatory cytokines (Ccl2, Ccl5, and Il6) that contribute to the pathogenesis of atherosclerosis (Nuutinen, Ailanen et al. 2018).

No data of an active local melanocortin system in human macro-circulation have been reported yet.

6. Role of the melanocortin system in cell proliferation and migration

The melanocortin system regulates migration and proliferation of different cell types, such as inflammatory cells, melanocytes, and melanoma cells, mainly by inhibiting them.

Pro-inflammatory cytokines, such as IL-1 and TNF-α, induce the expression of adhesion molecules (ICAM, VCAM, integrins, and E-selectin) on the endothelium, promoting monocyte diapedesis from vessels to peripheral tissues. α-MSH controls monocyte migration by downregulating the expression of adhesion molecules and regulating IL-8 and Gro-α secretion on human dermal endothelium through MC1R (Kalden, Scholzen et al. 1999). Consistently, α-MSH reduces T-lymphocytes (Cooper, Robinson et al. 2005), neutrophils (Catania, Gatti et al. 2004), and eosinophils (Raap, Brzoska et al. 2003) proliferation and migration.α-MSH and ACTH control melanocyte migration and have mitogenic and melanogenic effects (Swope, Jameson et al. 2012). On the contrary, melanoma cells overexpress MC1R and treatment with α-MSH *in vitro* slows down migration, opposing the effect of TNF-α (Zhu, Lalla et al. 2004). These studies indicate the involvement of the melanocortin system in the regulation of cell proliferation and migration in both physiologic and pathologic conditions.

EC and SMC proliferation and migration are hallmarks in vascular remodeling, neointima formation and atherosclerosis. The presence and implication of an active melanocortin system in human macrocirculation to control and prevent such processes that lead to cardiovascular diseases have never been studied yet.

HYPOTHESIS AND AIMS

We hypothesized that human macrovascular arteries express a local melanocortin system that could have a role in vessel homeostasis and in preventing and/or healing vascular remodeling.

The broader scope of this study was to identify new therapeutic targets to control maladaptive vascular remodeling.

The specific aims of the study were:

- To investigate whether human macrovascular endothelial cells (ECs) and vascular smooth muscle cells (SMCs) express any MCRs and/or other melanocortin system components
- To assess the *in vitro* effects of MCRs activation, and the underlying molecular mechanisms, on (*1*) aortic EC proliferation and migration and (*2*) aortic SMC proliferation, migration, and phenotypic switching
- To validate in human tissues the expression and localization of the MCRs.

MATERIALS AND METHODS

1. **Experimental plan**

Vascular remodeling is a complex process that involves different cell types, *i.e.* endothelial cells (ECs), smooth muscle cells (SMCs) and inflammatory cells. An injury in the endothelium leads to inflammatory process and SMC activation. EC migration rate and proliferation are essential features in repairing the vessel wall. SMC phenotypic switch, migration and proliferation contribute to vessel thickening and neointima formation. We tested all these processes by appropriate *in vitro* techniques that mimicked cell behaviors *in vivo* during vessel remodeling.

- Expression of MCS components in primary human aortic (HAoEC), coronary ECs (HCAEC) and human aortic smooth muscle cells (HAoSMC) were assessed by real-time PCR (qRT-PCR), Digital PCR (dPCR), western blot (WB), immunocytochemistry (ICC) and immunohistochemistry (IHC).
- To test whether the receptors are functional, intracellular cyclic adenosine-mono-phosphate (cAMP) concentrations were measured by ELISA.
- We tested the effects of MCR activation on EC migration with a gap closure assay and on EC proliferation measuring the ki67 expression by ICC. We then analyzed the pathways downstream MCR activation in migrating HAoECs by measuring intracellular EC Ca^{2+} concentrations, using a fluorescent Ca^{2+} indicator, and by genome-wide, time-dependent gene expression profiling, using microarrays.
- We assessed the effects of MCR activation on SMC migration by a gap-closure migration assay and a transwell migration assays along a chemoattractant gradient. We measured proliferation of SMCs by incorporation of a nucleoside analog in newly synthesized DNA. SMC phenotypic changes and associated biomarkers were visualized and measured by ICC and Real-Time PCR. Genome-wide gene expression profiling was performed in migrating HAoSMCs after stimulation with α-MSH in time-course experiments, using RNA sequencing. We finally tested the modulation of the p38 MAPK/HSF1 pathway, measuring p38 MAPK phosphorylation by WB.

2. **Human primary cells**

Human Aortic Endothelial cells (HAoECs) were purchased from ECACC (Salisbury, UK), Lonza (Allendale, NJ), and Promocell (Heidelberg, Germany). We obtained three adults HAoECs and

recoded them as c1 (from ECACC), c2 (Lonza), and c3 (Promocell). Likewise, we obtained three adult HCAECs and recoded them as c4 (from ECACC), c5 (Lonza), and c6 (Promocell). ECs were cultured following manufacturers' instructions. Cells were seeded in 75 mL plastic flasks (Corning, Tewksbury, MA) at a density of 2.5×10^3 cells/cm^2 in EGM Endothelial cells growth media (Lonza, Belgium). We purchased Human Aortic Smooth muscle cells (HAoSMC) from ECACC (Salisbury, UK) and we cultured them following manufacturers' instructions. Cells were seeded in 75 mL plastic flasks (Corning, Tewksbury, MA) at a density of 2.5×10^3 cells/cm^2 in SmGM-2 Smooth Muscle Growth Medium-2 (Lonza, Belgium). We performed all experiments at cell passages 4–7.

3. Chemicals

α-MSH was obtained from Phoenix Pharmaceuticals (Burlingame, CA); isobutyl methylxanthine (IBMX), PD0332991 isethionate, Thapsigargin, and Forskolin from Sigma-Aldrich (St. Louis, MO). The peptide 153N-6 (H-[Met5, Pro6, D-Phe7, D-Trp9, Phe10]-MSH$_{(5-13)}$) was bought from Bachem (Bubendorf, Switzerland), the peptide MSG 606, and JKC 363 from Tocris Bioscience (Bristol, UK). α-MSH, 153N-6, MSG 606 and JKC 363 were dissolved in water; IBMX, PD0332991, thapsigargin, and Forskolin in dimethyl sulfoxide (DMSO).

4. Genomic DNA sequencing

We used a 3500 Genetic Analyzer (Applied Biosystems) to perform DNA sequencing of MC1R gene ORF for all the cells type. Genomic DNA amplicons of the MC1R ORF were produced by PCR with the following primers: MC1R_Forward(1) (-25) 5'-TCCTTCCTGCTTCCTGGACA-3', MC1R_Reverse(1)(+980) 5'-CACACTTAAAGCCGCGTGCAC-3'. The amplified fragments were purified using the Agencourt AMPure XP kit (Beckman Coulter). Sequencing reactions were carried out using the BigDye Terminator v3.1 Kit (Applied Biosystems) in both strand directions to allow the production of four overlapping fragments. Sequencing primers used were the MC1R_Forward(1), MC1R_Reverse(1) and the inner MC1R_Forward(2) (+449) 5'-TGCGCTACCACAGCATCGTG-3' and MC1R_Reverse(2) (+510) 5'-CACCCAGATGGCCGCAAC-3'. Unincorporated fluorescent dideoxynucleotides and salts were removed with the BigDye XTerminator Purification Kit (Applied Biosystems). The purified sequencing reaction products were electrokinetically injected into a 50 cm Capillary Array filled with the POP-7 Polymer (Applied Biosystems). Electropherograms were analyzed by the Variant Reporter software v1.1 (Applied Biosystems).

5. Real-time PCR

Total RNA was extracted from ECs or SMCs, adding TRIzol Reagent (Invitrogen, Carlsbad, CA) directly to the culture dishes. Given that MCRs are single-exon intronless genes, we treated RNA samples with RNase-free DNase-I to eliminate genomic contamination and prevent amplification of genomic DNA. RNA quantification and purity assessment were performed by micro-volume spectrophotometry on an Infinite M200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland). RNA quality and integrity were checked by microfluidics electrophoresis with the RNA 6000 Nano Assay Kit on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Complementary DNA (cDNA) for single target gene expression analysis was synthesized from 1µg of total RNA for each sample using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR and single TaqMan Gene Expression Assays (Applied Biosystems) were used to assessing mRNA expression of the melanocortin receptors (*MCR*s), proopiomelanocortin (*POMC*), prohormone convertases, and SMC phenotypic markers, along with some reference genes as endogenous controls. Two replicates of each assay for each sample were run on a ViiA 7 Real-time PCR System (Applied Biosystems). Algorithms implemented in the ViiA 7 software v1.2 (Applied Biosystems) were utilized to impute experimental thresholds and baselines.

6. Digital PCR

Digital PCR (dPCR) was performed on a QuantStudio™ 3D Digital PCR System platform composed by the QuantStudio™ 3D Instrument, the Dual Flat Block GeneAmp® PCR System 9700 and the QuantStudio™ 3D Digital PCR Chip Loader (Life Technologies). dPCR was performed according to the manufacturer's instructions and the analysis was executed with QuantStudio® 3D Analysis Suite™ (Life Technologies). Primers labeled with FAM® dyes were used to evaluate the expression of target genes.

7. Antibodies

Primary antibodies for WB and ICC were: anti-MC1R rabbit polyclonal (Alomone Labs, Jerusalem, Israel; supplied with the specific control peptide antigen), anti-MC4R rabbit polyclonal (Alomone Labs, Jerusalem, Israel; supplied with the specific control peptide antigen), anti-ATP1A1 antibody rabbit polyclonal (Cell Signaling Technology, Danvers, MA), anti-β-actin (AC-15) mouse monoclonal (Novus Biologicals, Littleton, CO), anti-Caveolin-1

mouse monoclonal (R&D research, Minneapolis, USA). Antibodies against phenotypic protein markers were anti-α-SMA mouse monoclonal (Dako, USA), and anti-SM22α rabbit polyclonal (Abcam, UK). Antibody to measure cell proliferation was anti-Ki67 rabbit polyclonal (Abcam, Cambridge, UK). Antibodies to analyze the pathways were anti p38MAPK rabbit monoclonal (R&D research, Minneapolis, USA) and anti-phospho-p38MAPK (R&D research, Minneapolis, USA). Secondary antibodies for immunoblotting were donkey anti-rabbit and anti-mouse IgG conjugated, respectively, to IRDye 800CW and IRDye 680RD infrared dyes (LI-COR Biosciences, Lincoln, NE). Secondary antibodies for immunocytochemistry were donkey anti-mouse and anti-rabbit IgG conjugated to DyLight 649 or DyLight 594 fluorochromes (Jackson ImmunoResearch Laboratories, West Grove, PA).

8. **Immunoblotting**

HAoECs and HAoSMCs (1.2×10^6) were lysed in Milliplex MAP Lysis buffer (Millipore) with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) to obtain whole extracts, or with the FractionPREP Cell Fractionation Kit (BioVision, Milpitas, CA) to obtain plasma membrane extracts. Proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Thirty micrograms of each protein extract were mixed with the Novex Tris-Glycine SDS sample buffer 2× and the Novex sample reducing agent 10× (Invitrogen). Samples were loaded onto 4-12% gradient Novex WedgeWell precast Tris-Glycine polyacrylamide gels (Invitrogen) and run in Novex Tris-Glycine SDS running buffer for 40 min at 200 V. Samples were blotted on nitrocellulose membranes using an iBlot system (Invitrogen). Membranes were blocked in the Odyssey blocking buffer (LI-COR Biosciences) for 1 h. Pre-absorption was performed by incubating the anti-MC1R and MC4R antibodies for 30 min at room temperature with the inhibitory MC1R or MC4R peptide, respectively (two-fold excess of the peptide by weight). Primary or the pre-absorbed antibodies were diluted (1:1000) in the Odyssey blocking buffer (LI-COR Biosciences), and membranes were incubated overnight at 4°C. Anti-β-actin and anti-ATP1A1 antibodies (1:5000) were used as reference controls for whole or membrane extracts, respectively. Membranes were incubated with IRDye secondary antibodies (1:10000) for 20 min at room temperature. Immunoreactive bands were detected by an Odyssey Infrared Imaging System (LI-COR Biosciences).

9. **Immunostaining**

HAoSMC were plated in 8-chamber μ-slides (IBIDI, Martinsried, Germany) and fixed for 10 min with 4% paraformaldehyde solution. Non-specific antibody binding was prevented using a blocking solution of 10% normal donkey serum (Jackson ImmunoResearch Laboratories) for 1 h. Cells were incubated with the anti-MC1R, anti-MC4R and the anti-human/mouse/rat Caveolin-1 monoclonal (R&D research, Minneapolis, USA 1:100) overnight at 4°C, and then with DyLight-conjugated species-specific secondary antibodies (1:500) for 2 h at room temperature. In HAoEC proliferation experiments, cells were incubated with the anti-Ki67 primary antibody, using the aforementioned conditions. Slides were finally incubated with DAPI (Sigma-Aldrich, 1:1000) for 5 min, mounted in a fluorescence mounting medium (Dako, Glostrup, Denmark), and examined with an ApoTome fluorescence microscope (Carl Zeiss, Jena, Germany). Data were analyzed using the ZEN software v.5.0 SP1.1 (Carl Zeiss).

10. **Immunohistochemistry**

Formaldehyde-fixed paraffin 0.7 μm sections of a normal human aorta were obtained using a microtome (Leica RM 2145, Germany) and placed on glass slides. For immunostaining, slides were incubated with xylene and rehydrated by immersion in ethanol-series until rince with deionized water. After incubation with antigen retrieval (Dako, CA) at 96°C for 20 minutes, cooling and incubation with 3% hydrogen peroxide for 10 minutes, the PBS-washed slides were blocked with 5% BSA for 1 hour. Anti-rabbit MC1R and anti-rabbit MC4R (Alomone, Israel) 1:100 respectively diluted in PBS and 5% BSA, were applied O/N at 4 °C. Slides were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) and signals were revealed using the VECTASTAIN Elite ABC-HRP kit combined with the ImmPACT DAB EqV peroxidase (HRP) substrate (Vector Laboratories). Sections were washed with PBS, counterstained with hematoxylin for 2 seconds end rinced with deionized water. As control of the staining specificity, the anti-MC1R and anti-MC4R antibodies were pre-incubated 30 min with their specific blocking peptides.

11. **Intracellular cAMP**

Quantification of intracellular cAMP levels was performed using the cAMP ELISA kit (Enzo Life Sciences, USA). HAoECs and HAoSMCs were seeded to confluence in 96-well plates. Cells, prior to 5-min stimulation with α-MSH 10^{-8} M, were pre-treated for 30 min with IBMX 0.1 mM, to inhibit cAMP degradation by phosphodiesterases (PDEs). Forskolin was used ad positive

control. Cells treated with IBMX alone were used as controls. The concentration of α-MSH was selected based on previous publications on HDMECs (Hartmeyer, Scholzen et al. 1997; Scholzen, Brzoska et al. 1999) and pilot experiments that showed its effectiveness.

12. **Directional cell migration assay**

HAoECs were treated with a solution of 2.5µM 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindotricarbocyanine iodide (DiR, Biotium, Hayward, CA) in complete medium for 20 min at 37°C, washed and seeded onto 24-well plates with culture inserts (IBIDI). Inserts were removed to create a cell-free gap of approximately 500 µm, and HAoECs were allowed to migrate for 12h at 37°C and 5% CO_2 in the presence of 10^{-8} M α-MSH or in medium alone. As a control for receptor-binding specificity, cells were pre-treated with the MC1R-selective competitive α-MSH antagonist 153N-6 (10^{-5} M) for 15 min. Plates were scanned with the Odyssey imaging system (LI-COR Biosciences) at 0, 3, 9, and 12 h, at 84µm resolution and high-quality setting (emission, 800nm). Scans were converted to 8-bit images and analyzed with the NIH ImageJ software v1.38x. For time-course gene-expression analysis, 2×10^4 HAoECs were plated in high 35-mm dishes with culture inserts (IBIDI) and treated with α-MSH 10^{-8} M.

13. **Intracellular Ca^{2+} mobilization assay**

Intracellular Ca^{2+} levels were measured using the Fluo-4 NW Calcium Assay Kit (Invitrogen). HAoECs, seeded onto a 24-well plate with IBIDI culture inserts in a calcium-free medium, were loaded with 400 µL of Fluo-4 NW for 30 min at 37°C and 5% CO_2. Fluorescence was measured for 300 sec after treatment with α-MSH 10^{-8} M using an Infinite M200 PRO plate reader (excitation, 494 nm; emission, 516 nm). Thapsigargin (10^{-8} M) was used as positive control for $Ca^{2+}{}_i$ release, treating HAoECs for 120 seconds before stimulation with α-MSH (10^{-8} M). $Ca^{2+}{}_i$ changes were calculated as the difference between the area under the curve (AUC) before (resting levels) and after addition of stimuli.

14. **Time-course gene-expression analysis on HAoECs**

To isolate RNA from cells undergoing directional migration assay, we used the Agencourt RNAdvance cell v2 kit (Beckman Coulter, Beverly, MA), following the manufacturer's instructions. RNA extracted from migrating HAoECs at 0.5, 3, 6, and 12h was used for microarray analysis using the HumanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA). Raw data were extracted by Illumina GenomeStudio, imported in R v2.15.2 and analyzed with the *Lumi* package. Data were normalized by variance stabilizing transformation (VST) and

robust spline normalization (RSN). Probes with a detection *P*-value < 0.01 in <10% samples were filtered out.

To analyze the time-course microarray gene-expression experiments and the RNA sequencing data, we used the Short Time-series Expression Miner (STEM) v1.3.8 algorithm. Briefly, STEM implements a clustering method that lines up two steps. First, it selects a set of unique representative temporal profiles that, independently of the data, cover every possible expression profiles that can be generated in the experiment for a given set of parameters; second, it assigns to one of these temporal profiles only those gene profiles that pass the filtering criteria, as determined by a correlation coefficient. A permutation test was used to identify which profiles had a statistically significant enriched number of genes, and significant profiles were grouped into larger clusters by their correlation degree (≥ 0.7). The extent of the regulation is calculated as the maximum-to-minimum fold-change showed by the gene during the time-course.

Microarray data were validated investigating mRNA expression of 84 wound healing related genes at different time points by real-time RT-PCR, using the Human Wound Healing RT² Profiler PCR Arrays (Qiagen Sciences, Frederick, MD) following the manufacturer's recommendations. The concordance of microarray hybridization intensities (log_2 transformed) with PCR data (Ct) was measured by computing the Pearson correlation coefficient and assessing its statistical significance. Hierarchical clustering was performed using GENE-E v3.0.20 (https://software.broadinstitute.org/GENE-E). Analysis of functional relations among regulated genes was made using the DAVID Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov/home.jsp), testing for multiple annotations, *i.e.* Gene Ontology (GO) terms, KEGG pathways, and the Swiss Prot (SP)-Protein Information Resource (PIR) keywords. Redundant GO terms were removed using the web-based tool REViGO. A network map of the enrichment analysis was produced by the Cytoscape program v2.8.2 using the Enrichment Map app, a network-based visualization method for gene-set enrichment results.

15. **Radius migration assay**

Cell migration assays were performed using Radius™ 24-Well Cell Migration Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA) per manufacturers' protocol. HAoSMC were grown on the pre-treated Radius™ 24-well cell migration plate (Cell Biolabs, Inc.) at 1.0×10^5/well for 24 hrs. Cells do not attach to the Radius™ gel spot area (~0.68 mm). The day after plates were treated with Radius™ gel removal solution to expose the cell-free area to cell migration. After removal

of gel, cells were treated with the presence or absence of 10^{-10} M α-MSH to observe its effect on cell migration. Images were taken every 3 hours for 24 hours using a Zeiss ApoTome fluorescence microscope.

16. **Transwell migration assay**

For Transwell migration assays, harvested cells ($7x10^3$cells) were plated onto the upper chamber of a Transwell® Permeable Supports (Corning, Lowell, USA) with 8µm pores (Costar). 24 hours after we treated cells with specific compounds. At 48 hours cells were fixed with 4% paraformaldehyde in PBS, non-migrated cells on the upper side of the filter were removed with a cotton swab, and cells on the underside of the filter were stained with dapi. Images were captured using a Zeiss ApoTome fluorescence microscope. Relative cell migration was determined by the percentage of the migrated cells to the total number of the cells migrated in the control condition. For each experiment, the number of cells in nine random fields on the underside of the filter was counted, and two independent filters were analyzed.

17. **Proliferation assay**

We measured cell proliferation using the Click-it® EdU Microplate Assay (Thermo Fisher Scientific, USA). We plated 1000 cells /well in a 96 well plate and after 24 hours we treated them. At 48 hours, corresponding to cell doubling time, the assay was performed accordingly to the manufacturer instruction.

18. **RNA-sequencing of HAoSMC**

Two µg of total RNA were precipitated and poly(A)+ RNAs were enriched following MicroPoly(A) Purist kit protocol (Applied Biosystems). Libraries were prepared and pooled together using multiplex library RNA Barcoding reagents and Total RNA-Seq kits for the Sequencing by Oligonucleotide Ligation and Detection (SOLiD) System (Applied Biosystems). Complementary DNA (cDNA) amplification reaction was conducted with 20 µL template in 100 µl end-volume reaction and with 16 PCR cycles. Clonal amplification of library templates was performed on SOLiD p1 DNA beads by emulsion PCR (ePCR) using 0.5 pM library template and E120 EZbeads scale. Three different sample libraries were seeded in each lane of a SOLiD flow chip (400 million p2-EZBeads) and templates were single-end sequenced [50 base pairs (bp) forward] on a SOLiD 5500xl System (Applied Biosystems).

Using XSQTools (Applied Biosystems) with default parameters, we removed reads with low-quality base values and generated .csfasta and .QV.qual files for each sample. Using TopHat

v2.0.11 with Bowtie 1 (D. Kim et al., 2013; Langmead, Trapnell, Pop, & Salzberg, 2009) to handle color space reads, we mapped all reads to the human genome version HG38/GRCh38.76 downloaded from the Ensembl database (Flicek et al., 2014). We excluded 'haplotypes' and 'patches' sequences from the reference, in order to focus on primary assembly and to avoid under-estimation of gene expression. Then, we implemented the reference annotation based transcript (RABT) procedure, using Cufflinks suite v2.1.1 (Roberts, Pimentel, Trapnell, & Pachter, 2011; Trapnell et al., 2012), to create a new assembly for our downstream analysis, integrating information about known genes with those reads mapped in intergenic or intronic regions. Using the latter annotation, we identified novel transcripts and quantified them along with well-annotated genes. We estimated genes and transcripts expression levels by Cuffquant (using default parameters, apart from -multi-read-correct -frag-bias-correct 'reference.fasta', and -max-bundle-frags 100.000.000) and then Cuffnorm (with default parameters, apart from -total-hits-norm). For each feature, we computed both reads counts and fragments per kilobase of transcript per million fragments mapped (FPKM) values. Genes with an FPKM value ≥ 0.05 in at least 80% of samples were considered expressed. This threshold was obtained by correlating RNA-Seq data with those from a qPCR gene expression array (TaqMan Array Human Inflammation, Applied Biosystems) in preliminary experiments (not shown).

19. **Differential expression analysis on RNA-seq data**

Normalization procedures are a crucial step in the analysis of RNA-Seq data, since they may deeply affect the quality and quantity of differentially expressed genes. Here, we performed differential expression analysis controlling for "unwanted variation" (e.g. technical batch effects or other biological confounding variables) using the between-sample normalization method (R package RUVSeq) as proposed by Risso et al. (Risso, Ngai, Speed, & Dudoit, 2014). A set of empirical negative control genes, supposed not to be influenced by the biological covariates of interest (i.e. control and α-MSH-stimulated SMC), was used to estimate factors of unwanted variations (i.e. K parameter of the RUVs method). The number of k factors was chosen by comparing unadjusted with adjusted expression data by the use of diagnostic plots such as relative log expression (RLE) plots, scatter plot of first two principal components, and histogram plots of the distribution of the P values for testing differential expression between control and α-MSH-stimulated SMC. A k = 27 factors of unwanted variation was chosen in our

settings since it showed the best trade-off between data adjustment and the risk of data overcorrection.

The negative binomial generalized linear model (GLM) approach implemented in the edgeR package was used to perform differential expression analysis between control vs. α-MSH-stimulated SMC (Robinson, McCarthy, & Smyth, 2010). We deemed genes as significantly different at an FDR-adjusted P-value < 0.05.

20. **Statistical analysis**

Data passed the Shapiro-Wilk test and/or the D'Agostino-Pearson omnibus test for normality. Homogeneity of variances among groups was assessed with the Brown-Forsythe test and Bartlett's test: between-group variances were similar and standard deviations not significantly different. The within-group variance was estimated by computing mean square error. Differences among groups were compared using either one-way ANOVA followed by Tukey's multiple comparisons test, or two-way ANOVA (with treatment and time as factors) followed by Bonferroni *post-hoc* test, or two-tailed unpaired *t*-test, as appropriate. *P*-values < 0.05 were considered statistically significant. The analysis was done using GraphPad Prism v7 (GraphPad Software, La Jolla, CA).

RESULTS

Effects of MCRs activation on human aortic endothelial cells (HAoECs)

1. HAoECs constitutively express a functional MC1R, but not POMC

To determine which elements of the melanocortin system are expressed in human ECs from large vessels, we first performed a quantitative real-time PCR (qRT-PCR) for detecting specific mRNAs in six human primary cells, *i.e.* three aortic ECs (HAoECs) and three coronary artery ECs (HCAECs). All the macrovascular ECs clearly expressed MC1R, but no other known MCRs. Opposite to what was observed for human dermal microvascular EC (Scholzen, Brzoska et al. 1999), we did not detect POMC in HAoECs. We detected the prohormone convertase PCSK1 that process POMC into most of the derived peptides, but not PCSK2, which is needed to produce α-MSH (Seidah, Benjannet et al. 1999) (**Table 1**).

Cell	*MC1R*	*MC2R*	*MC3R*	*MC4R*	*MC5R*	*POMC*	*PCSK1*	*PCSK2*
HAoEC **c1, c2, c3**	++	–	–	–	–	–	+	–
HCAEC **c4, c5, c6**	++	–	–	–	–	–	+	–
HA	++	–	–	+	–	+	++	++

Table 1| **Expression of the MCRs, POMC, and prohormone convertases in primary human macrovascular endothelial cells** HAoEC: human aortic endothelial cells; HCAEC: human coronary artery endothelial cells; HA: human astrocytes (positive control). Three different primary lines for each type of EC were analyzed by qRT-PCR: from [c1,c4]ECACC, [c2,c5]Lonza, and [c3,c6]Promocell. Detection levels are reported as: –, undetected; +, < 35 Ct; and ++, < 30 Ct.

Given that MC1R is a highly polymorphic gene and that many variants are known to affect its signal transduction (Doyle, Fortin et al. 2012), we sequenced the MC1R open reading frame (ORF) of the six primary human artery ECs to identify and exclude from subsequent functional analysis those cells with gene variants that may interfere with the cellular response to MC1R ligands. All the three HCAECs bear a variant allele, whereas only two HAoECs did not present any polymorphism (**Table 2**). The variant alleles found have been associated with a decrease in cAMP production, in response to α-MSH stimulation (Xu, Thornwall et al. 1996; Schioth,

Phillips et al. 1999). We elected to use the HAoEC c2 cell line for functional tests due to its shorter doubling time.

Endothelial cell type	**SNP**	**Genotype**	***Position (nt)***	***Amino acid substitution***
HAoEC^{c1}	-	-	-	-
HAoEC^{c2}	-	-	-	-
HAoEC^{c3}	rs885479	GA	*1868*	*Arg163Gln*
HCAEC^{c4}	rs1805005	GT	*1558*	*Val60Leu*
HCAEC^{c5}	rs1805005	GT	*1558*	*Val60Leu*
HCAEC^{c6}	rs1805005	TT	*1558*	*Val60Leu*

Table 2 | **Mutational analysis of *MC1R* in primary human artery endothelial cells** HAoEC: human aortic endothelial cells; HCAEC: human carotid endothelial cells. Primary cell lines from ECACC(c1, c4), Lonza (c2, c5), and Promocell (c3, c6). Melanocortin-1 Receptor (MC1R) Ref. Seq. NM_002386.3.

To verify whether the three HAoECs express the MC1R protein, we performed an immunoblot analysis. We detected specific immunoreactive bands, corresponding to the molecular weight of the canonical fully active receptor (Sanchez-Laorden, Sanchez-Mas et al. 2006; Herraiz, Garcia-Borron et al. 2017), in total cell lysates and also in membrane extracts (**Figure 1.1A**), which showed that the MC1R receptor was expressed on the plasma membrane of the HAoECs.

We then measured the changes in intracellular cAMP levels after treatment with α-MSH, to test whether confluent HAoECs express a functionally active MC1R. Indeed, in cells grown to confluence in the presence of the phosphodiesterase inhibitor IBMX, a 5-minute stimulation with α-MSH 10^{-8} M induced a significant increase of intracellular cAMP (**Figure 1.1B**). Co-incubation with the MCR competitive α-MSH antagonist 153N-6 10^{-5} M (Chhajlani 1996) abolished the elevation of cAMP, indicating that MC1R is specifically activated by α-MSH (**Figure 1.1B**).

Finally, to test whether HAoECs express MC1R *in vivo*, we performed immunohistochemistry staining for the receptor in formaldehyde-fixed paraffin sections of a normal human aorta. We

observed a positive MC1R staining of endothelial cells, confirming the *in vitro* observations (**Figure 1.1C**).

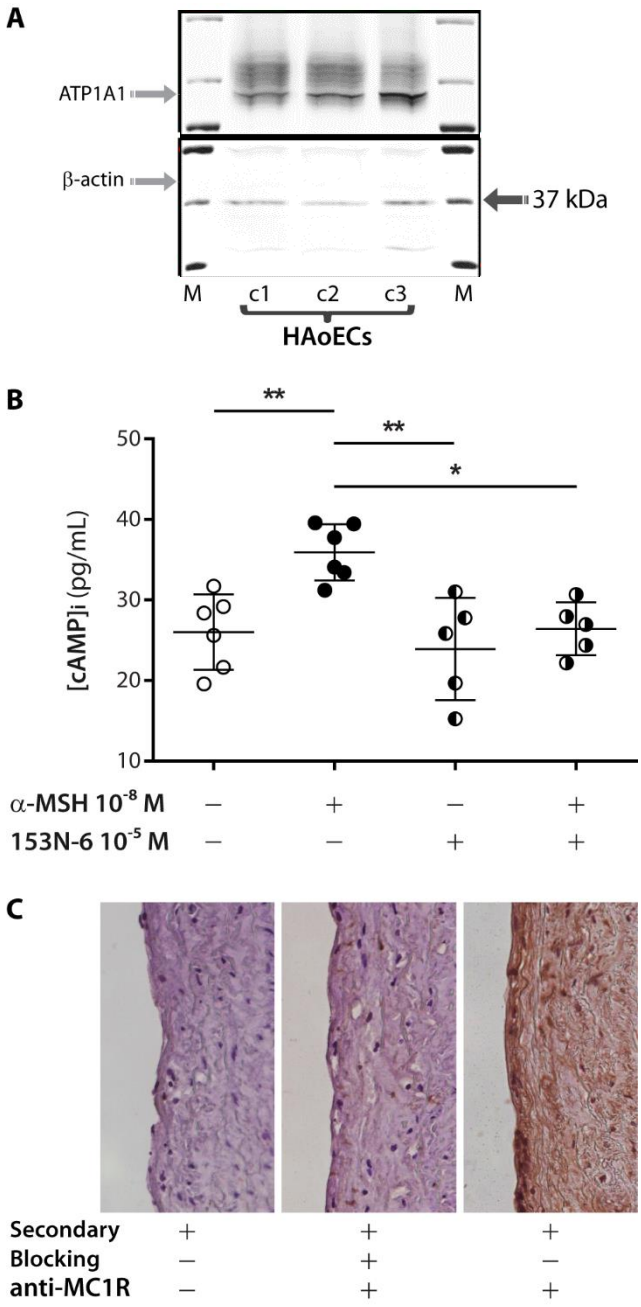


Figure 1.1 HAoECs express a functional MC1R (A) Immunoblot analysis of membrane extracts showed that all three studied primary cells express MC1R on the plasma membrane. A grey arrow (blot show below) indicates immunoreactive bands of 37 kDa specific for MC1R. The ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) protein (light grey arrow) was analyzed as a membrane-specific loading control, to confirm proper identification of the plasma membrane fraction (above). Lanes are: M, molecular weight marker; c1, c2, and c3 are 3 different primary HAoECs (c1 – from ECACC, c2 – Lonza, and c3 – Promocell). (B) Intracellular cAMP concentrations were measured in confluent HAoECs after treatment with α-MSH 10^{-8} M for 5 min, with or without the MC1R-selective competitive α-MSH antagonist 153N-6. Results are shown as box plots, representing the average of N = 5

independent replicates. The box bounds the interquartile range (IQR) divided by the median and Tukey-style whiskers extend to a maximum of 1.5 × IQR beyond the box. *P < 0.05, based on one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for preselected comparisons [F(3, 16) = 5.201, P = 0.0116]. (C) Immunohistochemical detection of MC1R in a normal human aorta specimen confirmed that HAoECs express the receptor in vivo (10×). To control for the specificity of the staining, we used the secondary antibody alone (left), the secondary antibody with the pre-adsorbed anti-MC1R antibody (center), and the secondary antibody with the anti-MC1R antibody (right): only the latter showed an intense, specific staining.

2. α-MSH promotes migration of HAoECs via MC1R activation

To determine whether MC1R activation has any influence on HAoECs migration and/or proliferation, we used a gap closure assay that measures directional cell migration. As shown in **Figure 2.1A**, stimulation with α-MSH 10^{-8} M enhanced HAoECs migration: in comparison with cells cultured in medium only, migration speed appeared to accelerate after 3 h of treatment and became significantly higher at 9 and 12 h in α-MSH-treated cells. Consistently, concomitant use of 153N-6 10^{-5} M was able to abolish the pro-migratory effect of α-MSH, whereas treatment with 153N-6 alone did not alter ECs migration speed (**Figure 2.1A**). Gap closure assays were also performed in the presence of the proliferation inhibitor PD0332991: as expected, blocking cell proliferation increased gap closure time, but the higher speed in α-MSH-treated cells confirmed the enhancement in cell migration after MC1R activation, which was still significant at 9 and 12 h (**Figure 2.1B**).

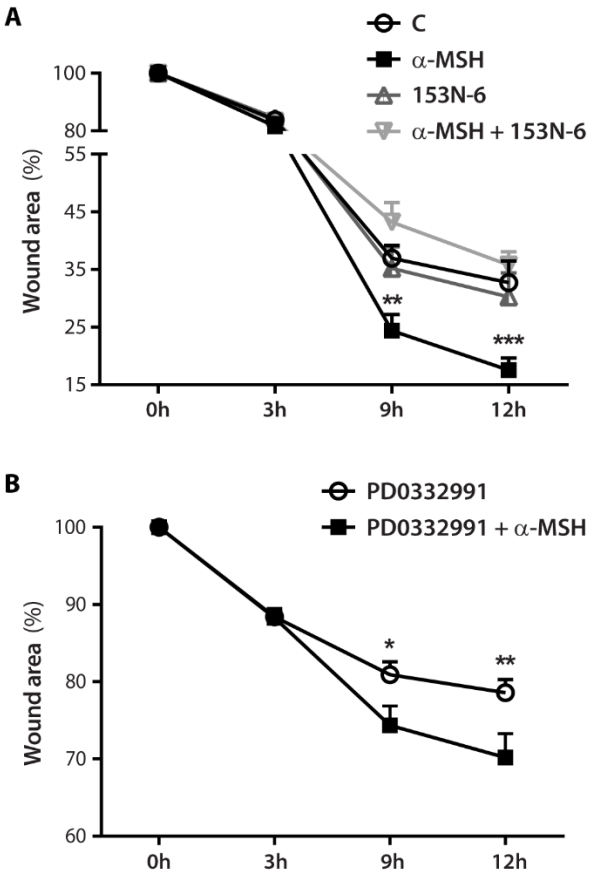


Figure 2.1 MC1R activation enhances HAoEC migration (a) after insert removal, HAoEC monolayers were treated with α-MSH with or without the 153N-6 antagonist and allowed to migrate for 3, 9, and 12 h: migration into the wound was quantified using DiR cell staining and near-infrared fluorescence scanning. Results are shown as means ± s.e.m. (N = 6). **P < 0.01 and ***P < 0.001 α-MSH vs. medium alone (C), based on two-way ANOVA (interaction Time × Treatment) followed by Bonferroni post-hoc test [F (9, 80) = 2.957, P = 0.0044; F (3, 80) = 10.85, P < 0.0001 for treatment effect]. (b) Using the same protocol as in panel B, we repeated the directional migration assay in the presence of the proliferation inhibitor PD0332991. Results are shown as means ± s.e.m. (N = 10). *P < 0.05 and **P < 0.01 α-MSH vs. medium alone (C), based on two-way ANOVA (interaction Time × Treatment) followed by Bonferroni post-hoc test [F (3, 72) = 3.018, P = 0.0353; F(1, 72) = 9.074, P = 0.0036 for treatment effect].

Conversely, no clear-cut effect of MC1R activation was observed on ECs proliferation, as documented by counting the number of Ki-67 positive cells, which were not significantly different between treated and untreated HAoECs (**Figure 2.2**).

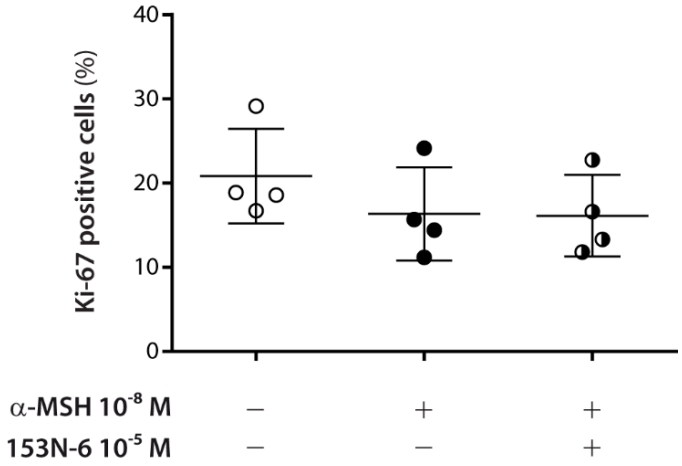


Figure 2.2 MC1R activation does not significantly influence HAoEC proliferation. Quantification of Ki-67 positive cells in separate experiments showed no significant differences between control and stimulated HAoECs. Data are expressed as an average percentage of Ki-67 positive cells, counting 10 different fields for each treatment conditions using an ImageJ tool. Results are shown as a scatter dot plot with mean ± SD (n = 4 independent experiments). P = n.s. by one-way ANOVA.

Since MC1R may signal through either cAMP increase or intracellular elevation of free cytosolic Ca^{2+} (Garcia-Borron, Sanchez-Laorden et al. 2005), we tested which signal transduction pathway was active in the enhancement of the HAoECs migration. Stimulation with α-MSH 10^{-8} M did not lead to an increase of intracellular cAMP in migrating HAoECs compared to control cells (**Figure 2.3**) On the contrary, MC1R activation resulted in a significant, rapid, and sustained increase in intracellular Ca^{2+} levels over the control, early after the removal of the insert in the cell migration assay (**Figure 2.4A**). This rise was almost completely abolished when HAoECs were pre-treated with the α-MSH antagonist 153N-6 10^{-5} M, which in turn alone did not affect Ca^{2+} signaling. Comparisons of the areas under the curve (AUC) confirmed that the α-MSH-induced rise in Ca^{2+} levels was highly significant (**Figure 2.4B**). Incubation with thapsigargin 10-8 M, a non-competitive inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) that causes a rapid rise of cytosolic Ca^{2+} by depleting endoplasmic reticulum stores (Lytton, Westlin et al. 1991), did not prevent a further significant rise of Ca^{2+} in response to a subsequent stimulus with α-MSH (**Figure 2.4C, 2.4D**). This was only partially inhibited by pre-treating HAoECs with the α-MSH antagonist 153N-6 10^{-5} M.

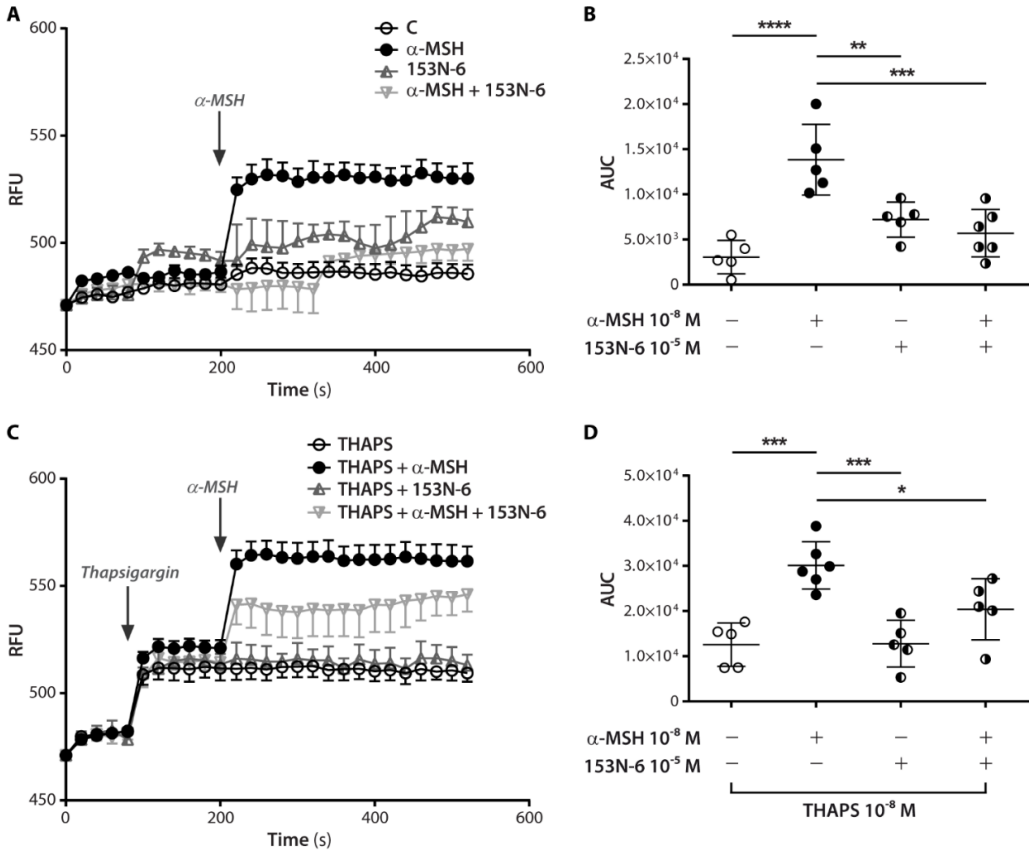


Figure 2.4 MC1R activation increases intracellular calcium levels in migrating HAoECs. Treatment with α-MSH 10^{-8} M after the removal of the insert in the cell migration assay induced a prompt increase in intracellular Ca2+ levels (a), which was completely abolished by pre-treatment with the MC1R-selective α-MSH antagonist 153N-6. Intracellular Ca2+ was detected using the Fluo-4 NW fluorescent calcium indicator. The rise of intracellular Ca2+ in response to the stimulus with α-MSH was not prevented by previous stimulation with thapsigargin (THAPS) 10-8 M (b), which is known to increase cytosolic Ca2+ by depleting endoplasmic reticulum stores. This was partially inhibited by pre-treating HAoECs with 153N-6. (a, c) Arrows indicate the time when either thapsigargin or α-MSH were added. Curves represent the means ± s.e.m. of five separate experiments. RFU, relative fluorescence unit. (b, d) Measurement of the areas under the curve (AUC) was used to compare α-MSH-induced effects with control treatments. AUC results are shown as box plots (N = 5 independent replicates per group), boxes are IQRs divided by medians, and whiskers are Tukey-style. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 in comparisons indicated by strait, horizontal lines, based on one-way ANOVA followed by Tukey's multiple comparisons test (b) [F(3, 16) = 14.56, P < 0.0001] and (d) [F(3, 16) = 15.43, P < 0.0001].

3. MC1R activation dynamically regulated several gene modules.

To evaluate the effects of MC1R activation at the transcriptional level, we assessed genome-wide gene expression profiles at 0.5, 3, 6, and 12 h after stimulation with α-MSH 10^{-8} M *vs.* control in HAoECs cultured in the directional cell migration assay. Applying stringent filtering parameters, we deemed expressed 18936 of the 47231 measured transcripts (40%).

Comparative time-course analysis, using the STEM algorithm (Ernst and Bar-Joseph 2006), identified 637 genes whose expression consistently showed a median change ≥ 30% over time as an effect of stimulation with α-MSH. These genes fitted 57 of the 625 possible model profiles computed by the clustering algorithm. Five hundred and six transcripts were associated with 15 distinct temporal profiles that showed a statistically significant enriched number of genes at an FDR < 0.05 (**Figure 3.1A**). The remaining 131 genes were associated with model profiles that had an FDR > 0.05 and, thus, were deemed as potentially arising from noise by random chance and excluded from subsequent analysis. Interestingly, we did not observe any significant change in MC1R expression level in migrating ECs after stimulation with α-MSH at any time point. The 15 significant temporal profiles of differential expression were grouped, based on their similarity by a correlation coefficient ≥ 0.7, to form 6 different clusters (**Figure 3.1B**). Overall, genes belonging to clusters 1 and 4 showed a marked increase in expression at 6 h in treated *vs.* untreated cells, whereas genes in clusters 2 and 3 displayed a marked decrease at the same time point; conversely, genes in clusters 5 and 6 appeared to be upregulated at 3 h.

A

Cluster	Profile	Genes	*P*-value
1	69	187	8.1×10^{-86}
	62	40	2.5×10^{-14}
	61	19	2.3×10^{-9}
	32	12	9.1×10^{-5}
2	71	79	1.8×10^{-47}
	65	30	6.6×10^{-11}
	72	19	3.2×10^{-10}
	100	8	2.9×10^{-3}
3	99	12	9.4×10^{-6}
	64	11	5.9×10^{-5}
	89	20	1.5×10^{-3}
4	33	22	8.2×10^{-12}
	41	19	3.0×10^{-3}
5	44	17	1.9×10^{-7}
6	70	11	1.3×10^{-4}

B

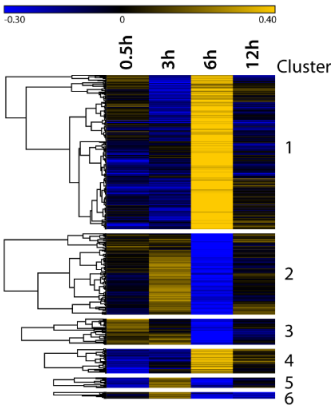


Figure 3.1 Significant temporal expression profiles and clusters of genes modulated by MC1R activation. The STEM algorithm attributed a number to each model profile and profiles were ordered based on the P-value significance of number of genes assigned vs. expected. Six clusters groups different model profiles based on their

similarity by correlation coefficient ≥ 0.7. (a) List of significant expression profiles ordered by significance (nominal P-value) within each cluster profile. The number of genes belonging to a profile is also reported. (b) Heatmap depicting the temporal expression profiles of genes within each cluster. Genes were hierarchically clustered into 6 groups using one minus Pearson correlation distance and the average linkage method. Data are expressed as the average log2 gene expression ratio of α-MSH stimulated cells to non-stimulated cells (N = 3 independent experiments per treatment group). Above the heatmap, the time scale (hours) of the time course experiments is reported. Globally normalized expression ratios are shown as a gradient color ranging from lower (blue) to higher (gold) values.

Time-course expression data of control and α-MSH stimulated cells at 3 and 6 h were validated using PCR-based arrays profiling key genes involved in wound healing. Forty-eight genes were detected by both microarray and real-time PCR (**Figure 3.2**), with a strong positive correlation between their average signal intensities (r ≥ 0.8, P < 0.0001 for all pairwise correlations).

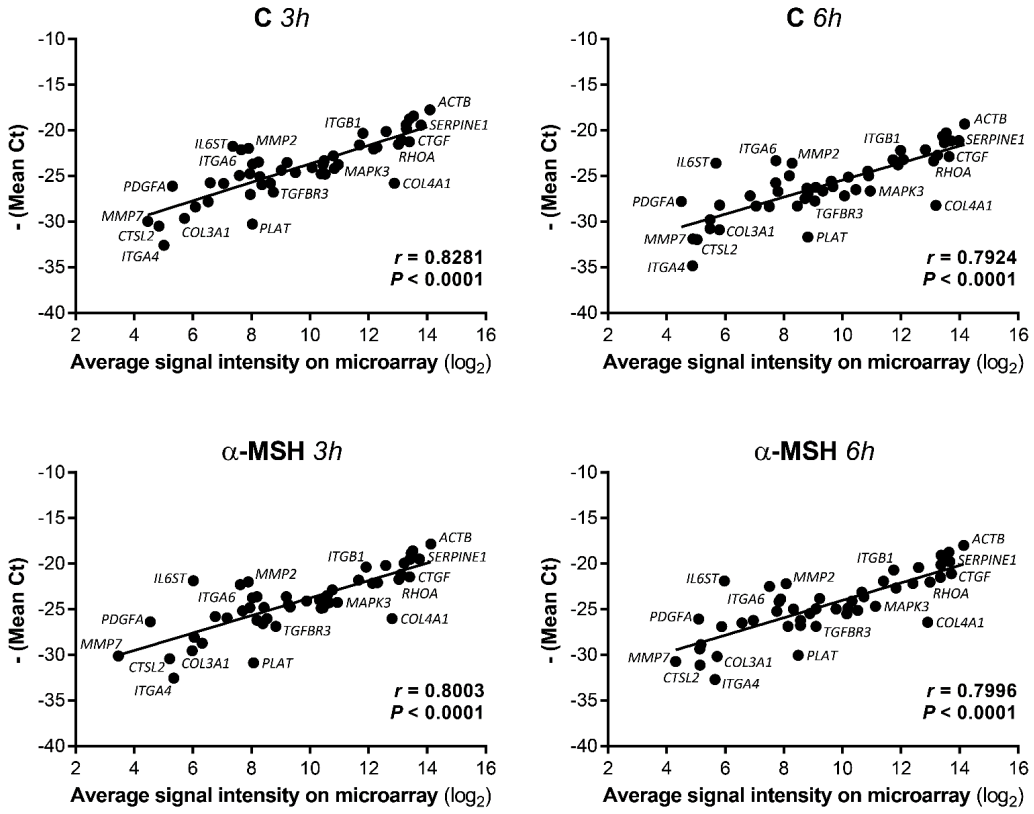


Figure 3.2 Validation of microarray data of non-stimulated and α-MSH-stimulated migrating human aortic endothelial cells (HAoECs) by real-time RT-PCR arrays. We performed a confirmatory analysis of gene expression data on migrating HAoECs at 3 and 6h, using Human Wound Healing RT² Profiler PCR Arrays (Qiagen). On the y-axis is the additive inverse mean Ct in control (C) and α-MSH-treated cells at a given time point; on the x-axis is the log2–transformed average raw signal intensity on microarray (N = 3 independent experiments per treatment group). We computed Pearson's correlation coefficients (r) and reported within the plots along with significance P-values. Plots show data of 48 genes detected by both microarray and real-time PCR.

To uncover the biological meaning beneath these transcriptional effects, we performed a functional enrichment analysis of the 506 regulated genes. Forty-four terms were significantly enriched at $P < 0.01$ and $FDR < 0.20$ and were used to draw a network to visually interpret biological data (**Figure 3.3**). The most significant gene sets included the phosphoprotein class ($P = 4.8 \times 10^{-8}$, $FDR = 1.9 \times 10^{-5}$), the endomembrane system ($P = 3.8 \times 10^{-5}$, $FDR = 1.3 \times 10^{-2}$), and the ECM-receptor interaction ($P = 2.3 \times 10^{-4}$, $FDR = 2.5 \times 10^{-2}$). Notably, 197 of the 506 regulated genes encode for phosphoproteins, 188 produce variant proteins by alternative splicing, and 65 are transcription factors or regulators. Importantly, 11 genes, *i.e.* AGRN, COL1A1, COL1A2, COL4A5, COL5A1, DAG1, ITGA2, ITGA10, LAMB1, LAMC1, and SPN, belonged to either the ECM-receptor interaction pathway or the extracellular matrix cellular component. To further analyze gene expression changes in a structured fashion, functionally enrichment analysis was performed associating annotated gene sets with the 6 different clusters of temporal profiles. Co-expressed gene subsets were visualized as temporal clustered profiles sharing functional annotations (**Figure 3.3**).

Coupling time-course gene expression analysis to enrichment analysis allowed identifying significantly regulated genes that have never been associated with MC1R signaling before, including genes involved in ECM-receptor interaction, vesicle-mediated transport, SNARE protein complex formation, and metal ion binding through metal-thiolate cluster structures (metallothioneins, MTs).

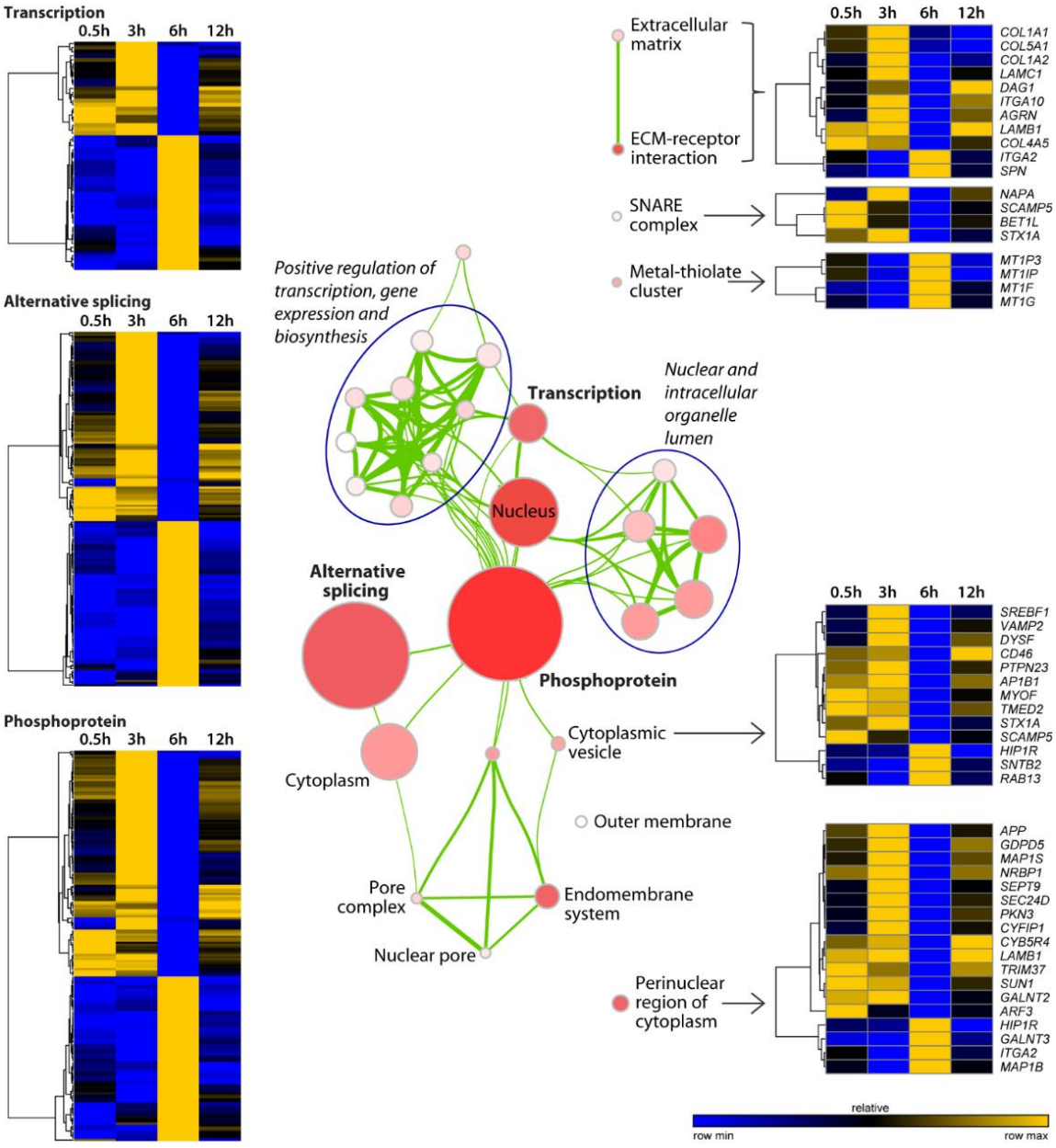


Fig. 3.3 Modules of co-regulated genes in migrating HAoECs upon MC1R activation. Upper left: the enrichment map of regulated genes in α-MSH-treated migrating HAoECs was drawn as a network of the most significant functionally annotated gene sets ($P < 0.01$ and Benjamini FDR < 0.20). Nodes represent gene sets. Enrichment significance is encoded by node color intensity gradient from lower (light red) to higher (dark red). Node size is proportional to the gene set size. Gene sets are connected based on their similarity by green edges, and edge thickness measures the degree of the overlap between two gene sets (calculated using a cut-off of 0.375 of the Jaccard plus Overlap combined coefficient). Clusters of tightly and functionally related gene sets were manually circled and assigned an overall label. Right and lower left: heat maps of temporal expression patterns of relevant gene sets and pathways are displayed. Hierarchical clustering of genes was performed using one minus Pearson correlation distance and the average linkage method. Row normalized expression values are shown as a gradient color ranging from lower (blue) to higher (gold) values.

Effects of MCRs activation on human aortic smooth muscle cells (HAoSMCs)

4. HAoSMCs constitutively express a functional MC1R, and MC4R but not POMC

To investigate whether HAoSMCs expressed any melanocortin system component, we first analyzed the expression of MCRs and POMC by qRT-PCR for detecting specific mRNAs. We found that HAoSMCs expressed both MC1R and, at a lower level, MC4R. We did not detect other known MC receptors, nor the melanocortin precursor POMC (**Table 3A**). We confirmed this observation using the digital PCR (dPCR) technique, measuring the exact number of MCR mRNA copies expressed by HAoSMCs cultured in control growth media (**Table 3B**). As for ECs, we sequenced the MCR open reading frame (ORF) of the primary human artery SMCs to identify and exclude from subsequent functional analysis those cells with gene variants that may interfere with the cellular response to MCR ligands (data not shown).

A

Cell	*MC1R*	*MC2R*	*MC3R*	*MC4R*	*MC5R*	*POMC*
HAoSMC	++	–	–	+	–	–

B

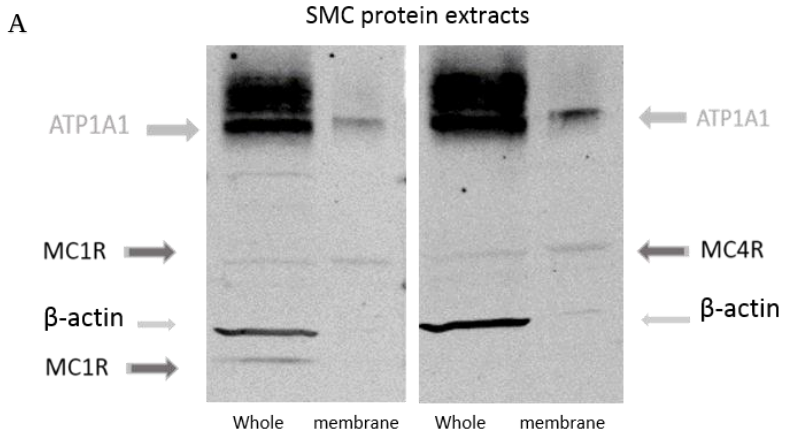
GENES	*Copies/µl*
MC1R	60.83
MC4R	31.22

Table 3 | Expression of the MCRs and POMC in primary human macrovascular smooth muscle cells
HAoSMCs express MC1R and MC4R. (a) MCRs and POMC mRNA expression were tested by qPCR. HAoSMCs express MC1R and MC4R at a lower level but not POMC. Detection levels are reported as –, undetected; +, < 35 threshold cycles (Ct); and ++, < 30 Ct (b) MC1R and MC4R mRNA expression was tested by PCR. The absolute quantitation for digital PCR results was reported as number of copies of mRNA per µ liters of samples loaded on a chip.

To verify whether HAoSMCs express the MC1 and MC4 receptors on the membrane, we performed a western blot and an immunofluorescence analysis. We detected by western blot

on total and membrane extracts the immunoreactive bands corresponding to the molecular weight of different isoforms of the MC1 and MC4 receptors. In detail, we could identify bands of 37kDa corresponding to the glycosylated monomeric form of MC1R in the total whole extract and a putative functional dimeric 58kDa band (Sanchez-Laorden, Sanchez-Mas et al. 2006) in both total and membrane specific extracts. We identified only the dimeric 58kDa form of MC4R, and not the monomeric form, in both total and membrane extracts. The 42kDa band of β-actin (positive cytoplasmic control) was visible only in the whole extract, thus assuring that the observed immunoreactive bands in the extracts are specific for the plasma membrane (**Figure 4.1A**).

We further showed by immunocytochemistry (ICC) that the MCRs co-localized with caveolin, a specific membrane protein (Rothberg, Heuser et al. 1992) (**Figure 4.1B**). MC1R and MC4R were clearly detectable on cell membranes and the membrane co-localization was measured using the Pearson's colocalization coefficients for all the images taken with the confocal microscope (**Figure 4.1C**).



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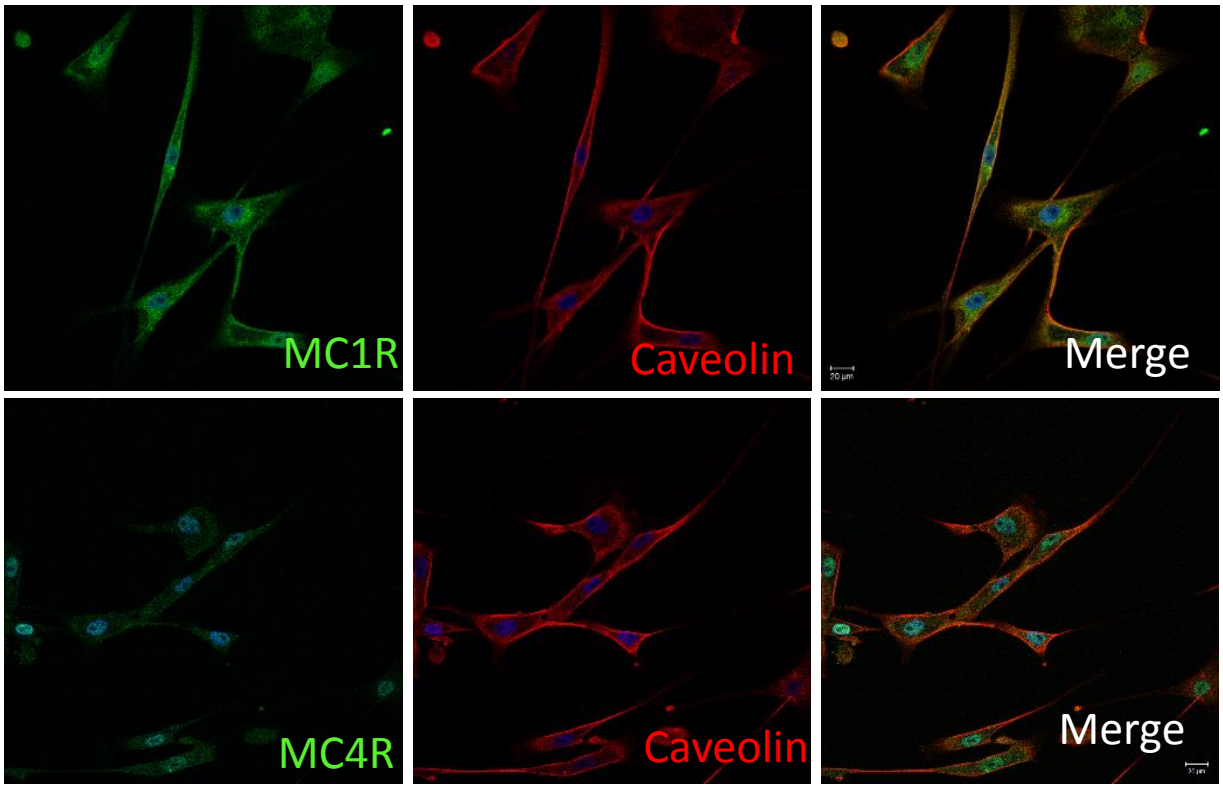

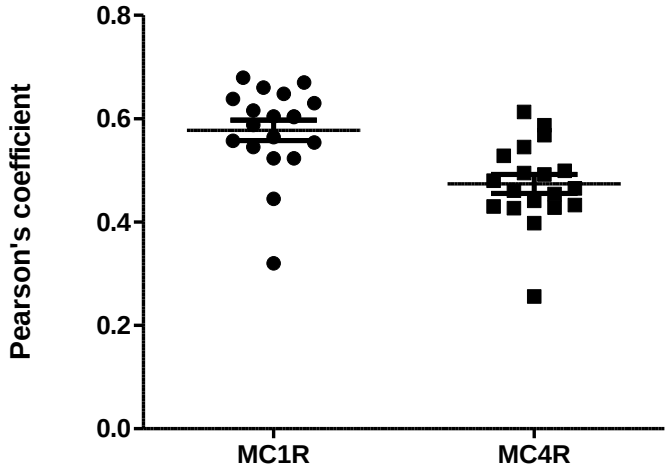

Fig 4.1 HAoSMCs express MC1R and MC4R on the membrane. (A) WB on whole and membrane protein extracts. Anti ATP1A1 antibody was used as membrane marker, beta-actin as a cytoplasmic marker. Bands of 37kDa and 58kDa were detected in the whole extract while only the dimeric form is expressed in the membrane. (B) MC1R and MC4R protein expression were tested by ICC using specific anti-MCR antibodies (green) and Caveolin (red) was used as membrane marker. (C) The graph showed the Pearson's co-localization coefficients for MC1R and MC4R with Caveolin measured on single confocal images from three independent experiments.

Finally, to test whether HAoSMCs express MC1R and MC4R *in vivo*, we performed immunohistochemistry staining for the receptors in formaldehyde-fixed paraffin sections of a normal human aorta. We observed a positive staining of SMCs and ECs for MC1R, and a positive staining of SMCs for MC4R, confirming the *in vitro* observations (**Figure 4.2**).

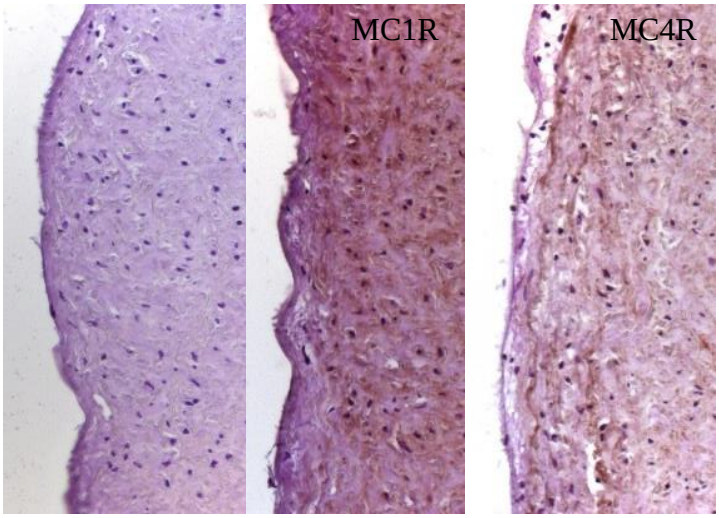

Figure 4.2 Immunohistochemical detection of MC1R and MC4R in a normal human aorta specimen confirmed that HAoSMCs express the receptor *in vivo* (10×). To control for the specificity of the staining, we used the secondary antibody alone (left); in the middle is shown in brown the positive staining for MC1R in the endothelium and in the tunica media; the slice on the right shows the MC4R expression in the tunica media.

MCRs are G-protein coupled receptors (GPCRs) associated with cAMP production, and so we measured the changes in intracellular cAMP levels after treatment with α-MSH, to test whether confluent HAoSMCs express functionally active MCRs. We observed that 5-minute treatment with α-MSH increased cAMP production and that α-MSH dose was inversely correlated with cAMP production. Indeed, concentrations of 10^{-10} and 10^{-12} M caused a significant rise in intracellular cAMP, in the presence of the phosphodiesterase inhibitor IBMX, compared to the control, non-treated cells (**Figure 4.3**). We chose the α-MSH concentration of 10^{-10} M to perform all subsequent experiments.

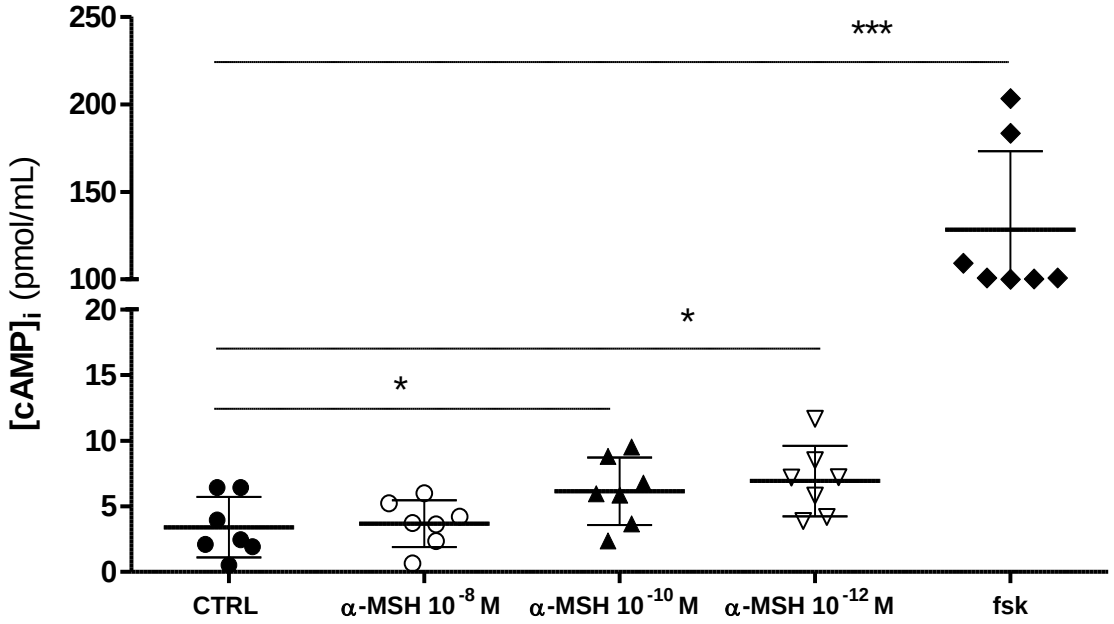

Figure 4.3. HAoSMCs express functional MCRs. The graph shows the increase in cAMP production after treatment with decreasing concentrations of α-MSH. We used scalar doses of α-MSH from 10^{-8} to 10^{-12} M. The latter concentration corresponds to the physiological concentration in human plasma. Forskolin (Fsk) was used as a positive control. The ELISA assay showed dose-dependent effects of α-MSH on MC1R activation (n=7). *p<0.05, ***p<0.001 vs. Ctrl; 1-way ANOVA followed by Bonferroni test.

5. The activation of MCRs modulates HAoSMC phenotypic changes

Morphology is the main parameter that defines the SMC phenotype. Contractile SMCs are elongated, spindle-shaped, whereas synthetic SMCs are less elongated and have a cobblestone morphology, defined as epithelioid or rhomboid (Owens, Kumar et al. 2004; Rensen, Doevendans et al. 2007). To determine whether α-MSH affects SMC morphology, we treated cells with a single-pulse of α-MSH $10^{-}10$ M for 48h: cell images were taken every 3h by an Apotome Zeiss microscope. We analyzed cells shape using an Image J v1.47 plug-in that automatically measures minor and major cellular axis. Treatment with α-MSH significantly promoted HAoSMCs elongation with peak effects at 6-12 h after treatment (fold difference of the number of elongated cells with respect to control = 2.6, p<0.01) (**Figure 5.1**).

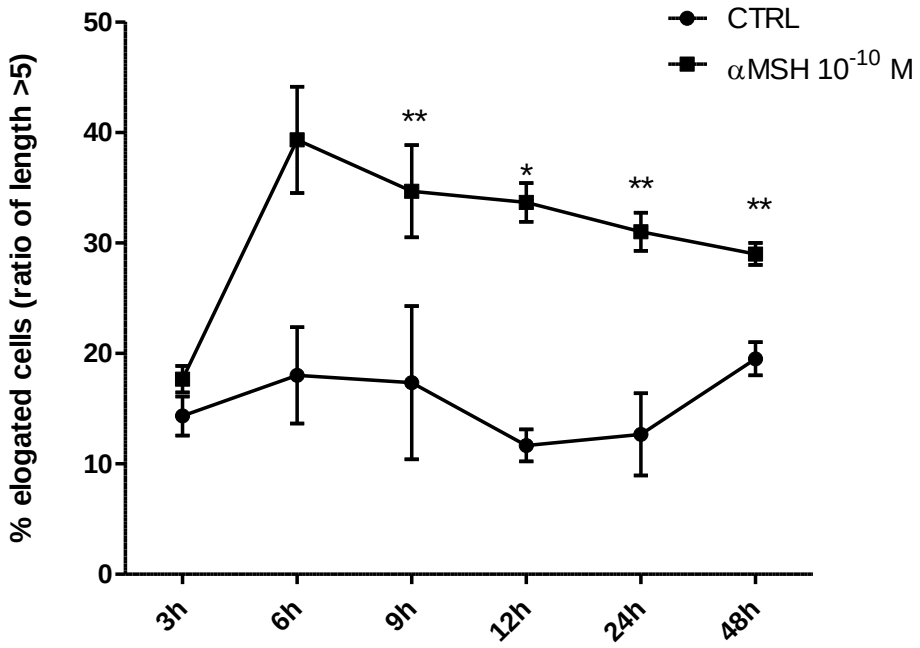

Figure 5.1 α-MSH promotes HAoSMC spindle-shape contractile morphology. Comparison of the ratio of pole-to-pole length (long axis/short axis) between cells. The graph represents a time course analysis of the number of cells with a higher than five ratio of length. *p<0.05, **p<0.01.

To confirm the phenotypic modulation of HAoSMCs, we analyzed by qRT-PCR the expression level of smooth muscle α-actin (*ACTA2*), smooth muscle myosin heavy chain (*SM-MHC*), calponin (*CNN1*) and SM22-alpha (*TGLN*), which are the most representative markers of the contractile phenotype (Rensen, Doevendans et al. 2007; Beamish, He et al. 2010). These genes were more expressed in HAoSMCs treated with α-MSH 10^{-10} M compared with untreated cells (**Figure 5.2A**). Immunocytochemistry imaging of HAoSMCs stained with anti-smooth muscle α-actin (α-SMA) and SM22-α were consistent with the observed changes in cell morphology after treatment with α-MSH and confirmed the increased protein expression of the typical markers of the contractile phenotype (**Figure 5.2B**).

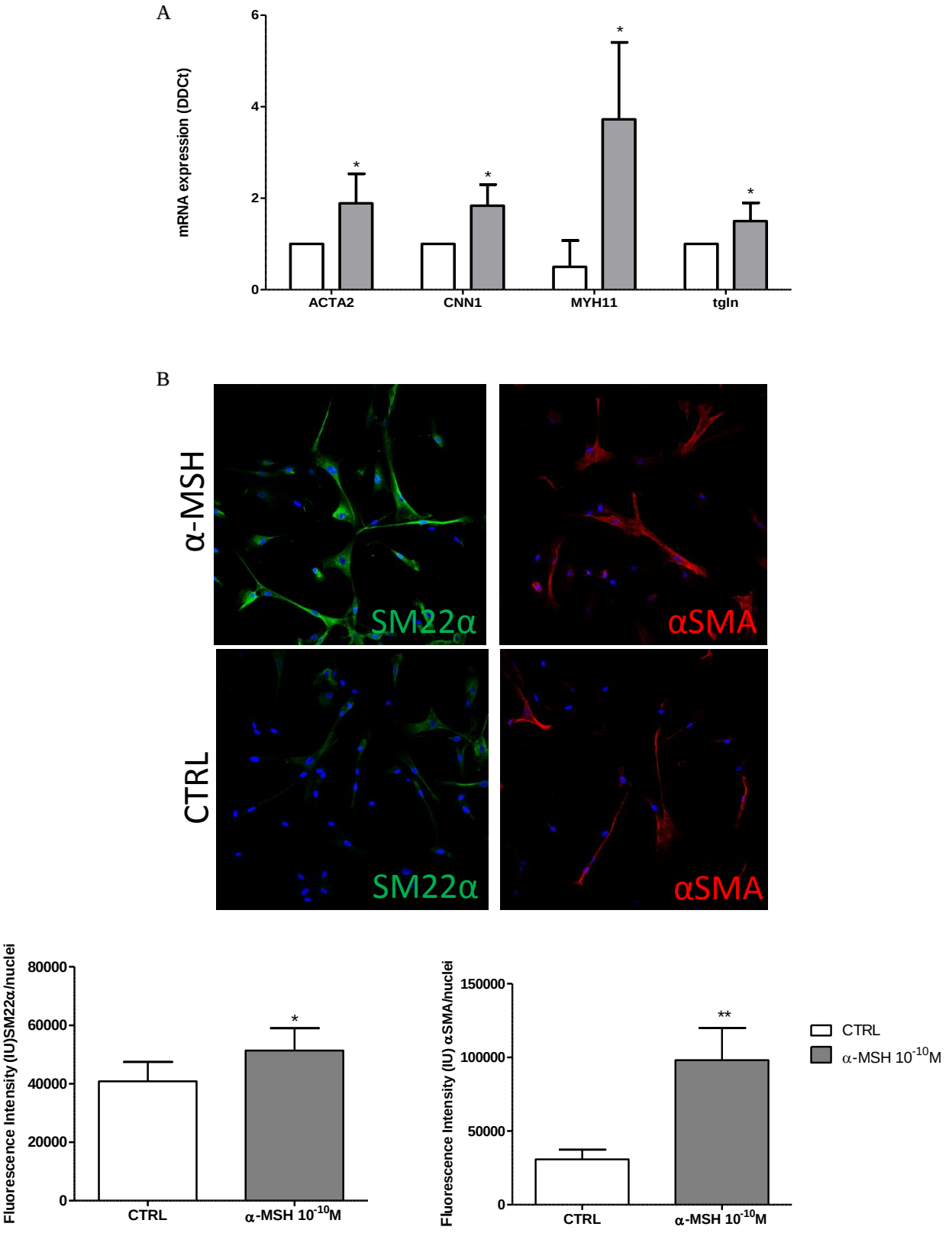

Fig. 5.2 α-MSH is able to modulate HAoSMCs phenotypic switching from a synthetic to a contractile form. (**a**) Gene expression analysis of markers of the contractile phenotype; the graph shows the DDCt values over the control of *ACTA2, SM-MHC, CNN1,* and *TGLN*. Mean ± SD, n=5. *p<0.05, **p<0.001 vs. control. (**b**)

Immunofluorescence staining for α-SMA(red) and SM22α, (green), confirming the phenotypic changes. Fluorescence quantification indicated that treatment with α-MSH significantly increased α-SMA and SM22-α expression. Fluorescence intensities were normalized on nuclei number counting. Mean ± SD n=4, **p<0.001, ***p<0.0001 vs. control.

6. Activation of MC1R modulates HAoSMC migration with mild effects on proliferation.

SMC phenotypes commonly show differences in the rate of proliferation and migration (Rensen, Doevendans et al. 2007): elongated, contractile cells migrate and proliferate less than the synthetic ones. To determine the effects of α-MSH on HAoSMC migration, we performed two different types of assay: a gap-closure assay for directional migration and a modified Boyden chamber assay through a gradient of PDGF-BB for chemotaxis. We performed a time course experiment by analyzing gap closure every 3 h for 12 h. α-MSH treatment slows down HAoSMC migration with the maximum effect between 6 and 9 h (mean fold difference = 4.9, p<0.001). Pre-treatment with the proliferation inhibitor PD0332991 did not abolish α-MSH-mediated acceleration in gap-closure, confirming that MCR activation had specific effects on migratory pathways (**Figure 6.1**).

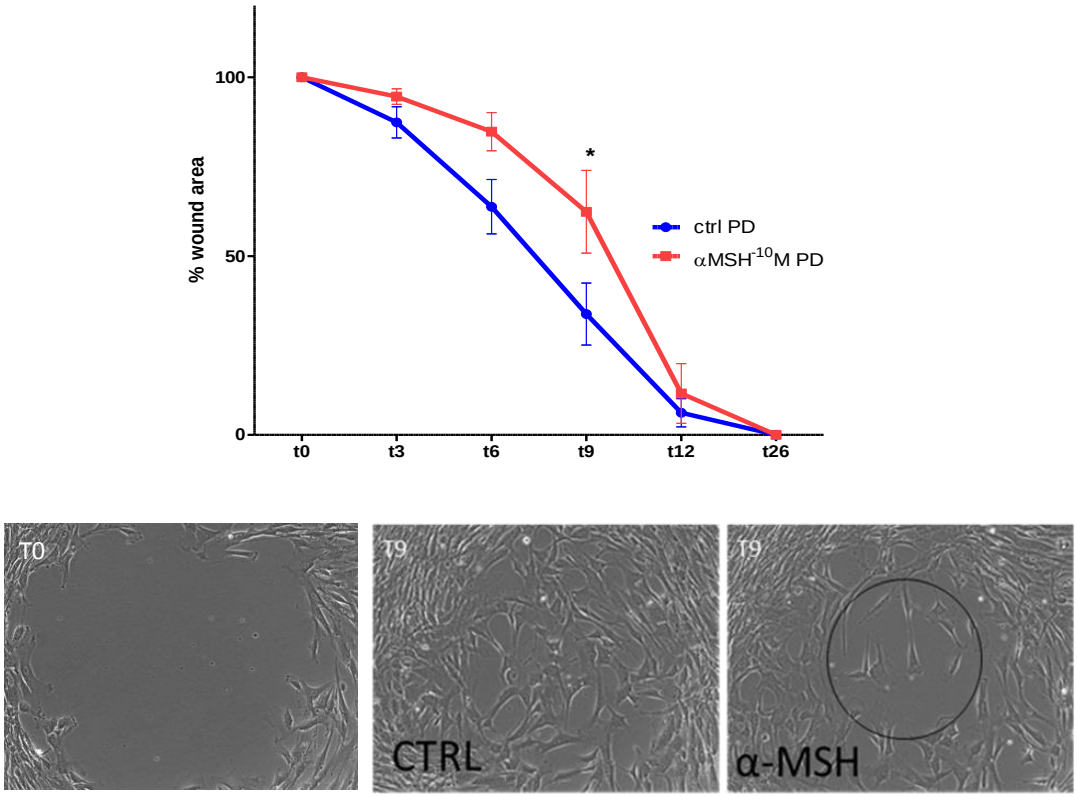

Figure 6.1. **MCR activation slows down HAoSMC migration.** (a) Wound healing time lapse migration assay show a slow in cells migration in treated cells compared to the control in presence of the proliferation inhibitor

PD0332991. *p< 0.05 vs Ctrl; 1-way ANOVA followed by Bonferroni test. (b) Representative images of gap closure. At 9h after α-MSH treatment, there is a significant difference over the control in the percentage of closure.

Likewise, in the transwell migration assay treatment with α-MSH reduced the number of migrating cells (mean fold difference = 1.95, p<0.01). Treating the cells with the MC1R specific inhibitor MSG606 (Cai, Stankova et al. 2013) reverted the effect. Instead, the treatment with JKC-363, the most selective and potent MC4R inhibitor (Kim, Small et al. 2002; Verty, McFarlane et al. 2004), was not able to contrast α-MSH-dependent reduction in migration, suggesting that the effect was due to MC1R activation (**Figure 6.2**).

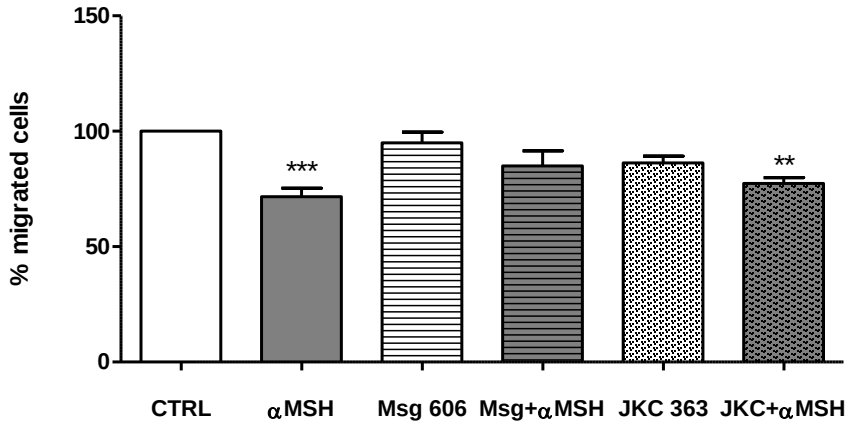

Figure 6.2. **MC1R activation slows down HAoSMC migration**. (a) The graph shows the percentage of migrated cells in treated and untreated conditions reduced the number of migrating cells (mean fold difference 1.95, p<0.01) and treating the cells with the MC1R specific inhibitor MSG606 reverted the effect, opposite JKC-363, the MC4R inhibitor didn't contrast α-MSH effect.***p< 0.001 vs Ctrl; 1-way ANOVA followed by Bonferroni test.

We finally analyzed the role of MCRs activation on HAoSMC proliferation by a modified BrdU-incorporation assay. We treated cells with increasing doses of α-MSH for 24 hours and we showed that nanomolar concentrations of the melanocortin peptide were able to slightly but significantly reduce HAoSMCs proliferation (mean fold difference = 1.2, *p*<0.05) (**Figure 6.3**).

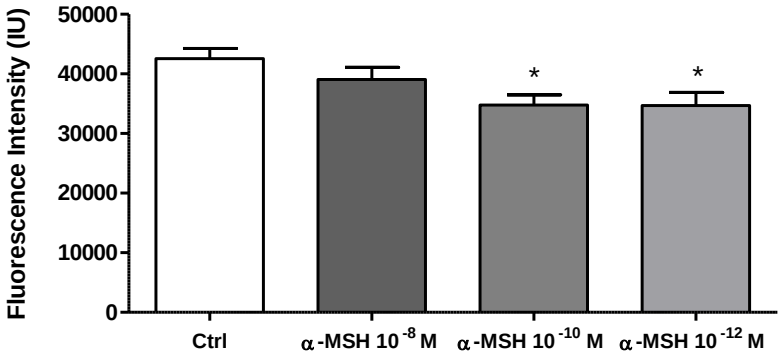

Figure 6.3 MCR activation reduce HAoSMC proliferation. BrdU proliferation assay indicated that α-MSH had a mild, but significant effect on cell proliferation (mean fold change 1.2, $p<0.05$). Mean ± SD, *$p<0.05$ vs. control.

7. α-MSH reduces HAoSMC cell migration modulating the p38MAPK/HSF pathway

To identify which pathways were activated after α-MSH administration, we performed a time-course RNA sequencing analysis of the transcriptome of HAoSMCs. We detected as expressed 12172 genes and we performed an analysis on the differentially expressed genes at 3, 6, 9 and 12 hours after α-MSH treatments. We obtained a list of genes significantly up and down regulated after α-MSH treatment grouped in time course profiles (data not shown). We chose to focus on the genes differentially expressed at 6 hours since this was the time preceding the maximum difference in cell phenotype and migration. We then performed a Gene Set Enrichment Analysis to identify the clusters of modulated genes that correlated with biological and functional pathways. Using Cytoscape and the Enrichment Map software, we generated a representative network of the positive or negative regulated clusters that are associated with biological processes (**Figure 7.1**). The map showed that α-MSH treatment negatively regulated pathways involved in the regulation of SMC migration and proliferation, in wound healing, and in extracellular matrix organization (blue nodes, bold-highlighted labels). These data support the results of the phenotypic assays and suggest that α-MSH reduces SMC migration and proliferation promoting the contractile phenotype.

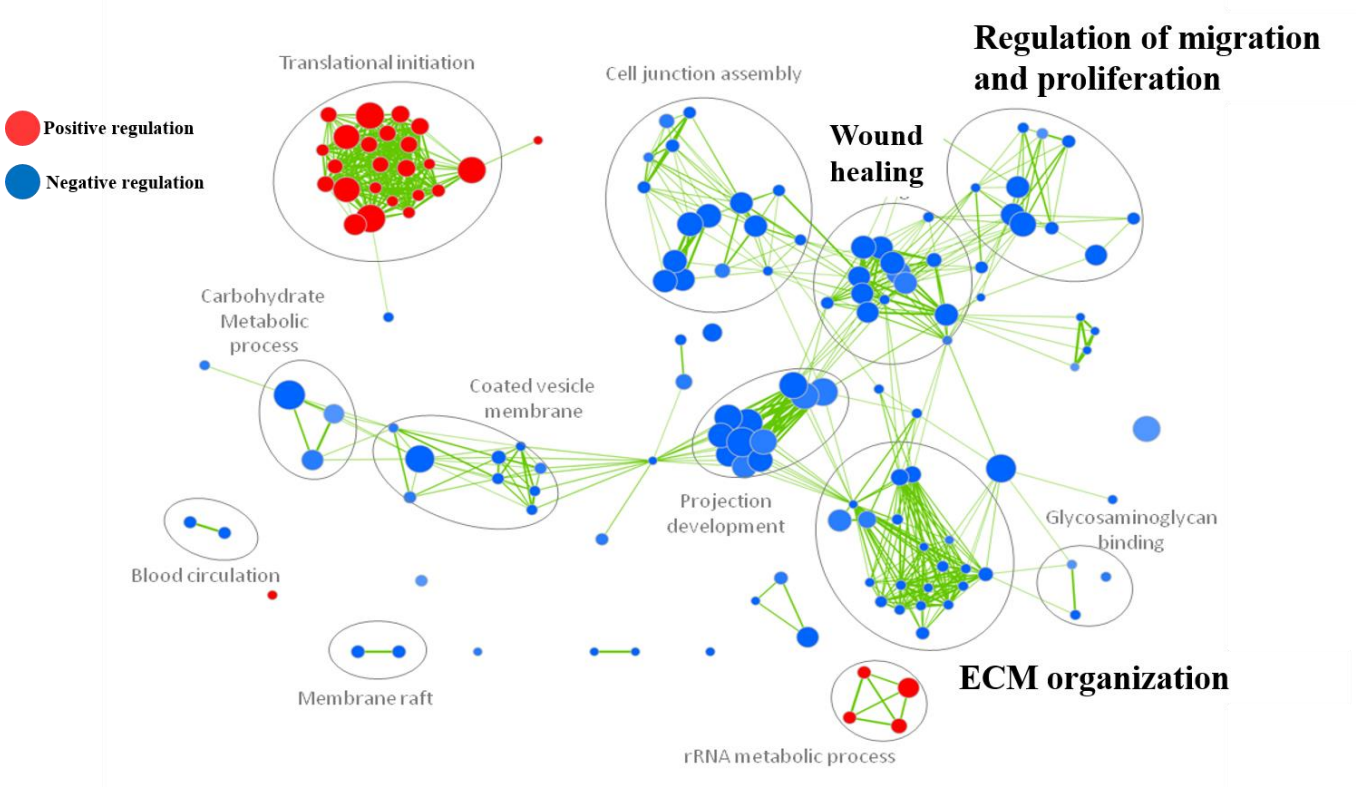

Figure 7.1 The enrichment map of regulated genes in α-MSH-treated HAoSMCs was drawn as a network of the most significant functionally annotated gene sets (P < 0.01 and Benjamini FDR < 0.20). Nodes represent gene sets. Enrichment significance is encoded by node color intensity gradient from lower (light red, blue) to higher (dark red, blue). Node size is proportional to the gene set size. Gene sets are connected based on their similarity by green edges, and edge thickness measures the degree of the overlap between two gene sets (calculated using a cut-off of 0.375 of the Jaccard plus Overlap combined coefficient).

Finally, we performed a Gene Set Enrichment Analysis to identify significant gene sets that were putatively associated with specific transcriptional factors. We found three gene clusters under the control of specific transcriptional factors significantly modulated by the α-MSH treatment (**Table 4**). In particular, we found genes under the control of Heat shock factor-1 (HSF1), which is known to regulate cell migration (Salinthone, Tyagi et al. 2008); SRY-Box 9 (SOX9), which regulate vascular smooth muscle cell differentiation (Xu, Ji et al. 2012); and NK2 Homeobox-5 (NKX2.5), which is involved in the regulation of Collagen Type I (Ponticos, Partridge et al. 2004).

NAME	SIZE	Enrichment Score (ES)	Normalized ES	FDR q-val (adj.p-value)
HSF	139	-0,194	-2,696	0,018
SOX9	171	-0,174	-2,640	0,012
NKX25	148	-0,168	-2,377	0,053

Table 4 | **Transcriptional factors that control set of genes negatively modulated after α-MSH treatment**. The table reports the three gene sets under the control of the cited transcriptional factors that are modulated after α-MSH treatment. In columns are reported the gene set size, the enrichment score (ES), the normalized ES and the adjusted p-value.

HSF1 is a substrate for p38 Mitogen-Activated protein kinases (p38MAPK) (Dayalan Naidu, Sutherland et al. 2016) and it is known that p38MAPK activation drive cell migration in vascular SMC (Kavurma and Khachigian 2003). Therefore, we treated cells for 5, 10, 15 minutes with α-MSH and we showed by WB that p38 phosphorylation was decreased by MC1R activation in a time-dependent manner (**Figure 7.2 A-B**).

These data suggest that a negative modulation of the p38MAPK/HSF1 pathway is a key mechanism of action underlying MC1R control on SMC migration (see **Figure 7.3** below).

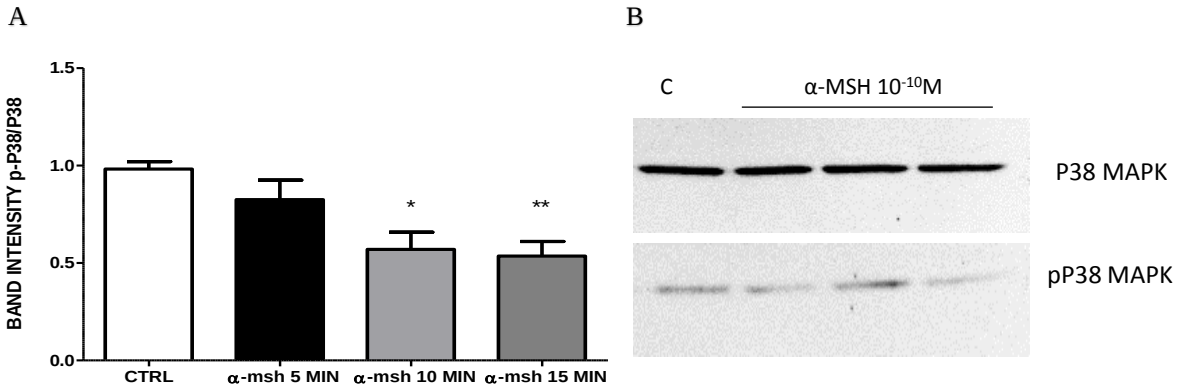

Figure 7.2 MC1R activation modulates HAoSMC migration through the p38MAPK/HSF pathway. (**a**) WB relative quantification of the bands of p38MAPK and phospho-p38MAPK. Each bar represents the mean of three different experiments per condition (Mean ± SD, *p<0.05, **p<0.001 vs. control, n=5). (**b**) Representative WB image of p38MAPK reduction in phosphorylation after treatment with α-MSH for 5/10/15 minutes.

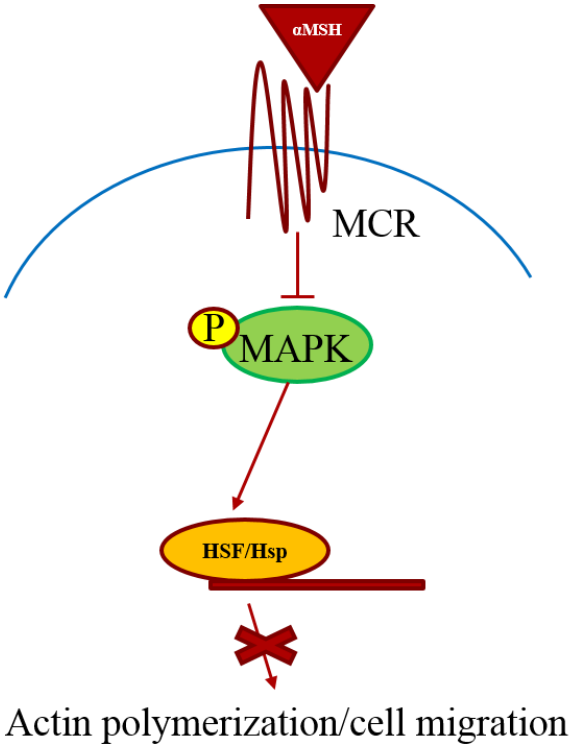

Figure 7.3. Cartoon of MCR mechanism in controlling SMC migration

DISCUSSION

Maladaptive vascular remodeling is one of the primary cause of artery dysfunction and/or occlusion. We provided evidence for a new function of peripherally expressed melanocortin receptors, *i.e.* regulation of EC and SMC motility, which may contribute to vessel homeostasis and the control of cellular processes that lead to maladaptive remodeling. Our study opens new possibilities to treat/prevent those cardiovascular diseases where remodeling plays a major role.

This work showed that human macrovascular ECs and SMCs constitutively express functional MCRs. In particular, human aortic ECs express MC1R, and human aortic SMCs express MC1R and MC4R. We showed that activation of MC1R drives faster ECs migration in an *in vitro* wound healing model, by enhancing intracellular Ca^{2+}. Time-course gene expression analysis allowed us identifying downstream molecular pathways associated with the enhanced ECs motility following α-MSH engagement of the MC1R. On the other hand, we showed that treatment with α-MSH promoted the switch from synthetic to contractile phenotype, slowed down migration, and reduced proliferation of human aortic SMCs. Consistently, we showed a time-dependent gene expression modulation of pathways associated with SMC migration and proliferation. We found that p38MAPK phosphorylation and HSF1 expression decreased after α-MSH treatment, and we propose a model in which MC1R activation controls aortic SMC migration through the p38MAPK/HSF1 pathway.

To our knowledge, this is the first demonstration that human macrovascular ECs and SMCs express MCRs and modify their phenotype and behaviors in response to their natural ligand, α-MSH. These observations revealed that the MCRs expressed peripherally in the human macrovasculature could be a target to treat and/or prevent maladaptive remodeling and related conditions.

Constitutive expression and cell-type restricted function of MC1R in HAoECs

We detected both the MC1R mRNA and protein in HAoECs and demonstrated that it was functionally active, since resting confluent cells were able to produce cAMP in response to exogenous α-MSH. No other component of the MC system was detectable in HAoECs. Consistently, we did not detect α-MSH in cell culture supernatants. Thus, HAoECs are not a source of MC peptides but may be targeted by endocrine secretion (by the pituitary gland) or

paracrine release of endogenous agonists (*e.g.* by immune cells at injured sites) (de Angelis, Sahm et al. 1995; Rensen, Doevendans et al. 2007). These findings represent a peculiar difference between macrovascular and microvascular ECs, as it has been reported that HDMECs express POMC and release MC peptides upon stimulation (Scholzen, Brzoska et al. 1999). In addition, unlike the microvascular ECs (Hartmeyer, Scholzen et al. 1997), MC1R expression did not significantly change upon exposure of the HAoECs to α-MSH. This finding suggests that in macrovascular ECs MC1R expression levels are not influenced by pathways that depend on its activation and that HAoEC migration is not dependent on MC1R upregulation. Considering EC heterogeneity, these results underline that artery ECs present cell type-restricted gene expression, which may account for a specific physiological function for MC1R, other than anti-inflammatory actions. This hypothesis led us to investigate whether MC1R activation could affect macrovascular EC migration after injury.

MC1R activation on HAoECs drives migration through the Ca^{2+} signaling cascade

EC migration is a fundamental process primed by damage and involved in vascular homeostasis and repair (Al-Soudi, Kaaij et al. 2017). Our data revealed that MC1R activation by α-MSH increased the rate of HAoEC migration, both in gap-closure and in injury-induced wound-healing assays, without significantly affecting cell proliferation. This effect was specifically induced by MC1R activation, since the 153N-6 peptide antagonist at MC1R abolished the α-MSH-driven migration of HAoECs, reverting it to the same rate of control cells. Conversely to our observations, it has been recently reported that α-MSH inhibits *in vitro* migration of HUVECs (Weng, Huang et al. 2014); but this reinforces our idea that EC are heterogeneous and the pro-migratory effect of α-MSH via MC1R activation is restricted to arterial ECs of the macrovasculature. EC migration is an essential step in wound closure after injury (Li, Huang et al. 2005; Michaelis 2014), and increased migration could promote the faster repair of the lesions.

Interestingly, MCRs can signal through an intracellular cAMP increase or transient intracellular elevation of cytosolic free Ca^{2+} (Bhasin, Yuan et al. 2010). In migrating HAoECs, α-MSH elicited a prompt elevation of intracellular Ca^{2+} but not of cAMP, suggesting that MC1R activation drives EC migration through the Ca^{2+} signaling cascade. This is coherent with the known effect

of Ca^{2+} mobilization on EC migration (Cines, Pollak et al. 1998). Ca^{2+} gradients regulate cell-ECM interactions responsible for the asymmetry between the cell front and rear adhesions, finally resulting in cellular directional movement (Li, Huang et al. 2005). Consistent with this, we observed a dynamic co-regulation of molecular pathways and cellular processes that appear to drive the faster closure of the wound (Cines, Pollak et al. 1998; Otsuka, Finn et al. 2012). Our finding suggests that MC1R activation promotes HAoEC migration mostly by regulating interactions between cell membrane integrin receptors and dystroglycan with their ECM counterparts, *i.e.* collagens, laminins, and agrin. Integrins and interstitial collagen influence directional migration, and this may be a key effect of the MCS in repairing large vessels.

MC1R activation in migrating HAoECs dynamically regulates expression of genes that control cellular trafficking and molecule mobilization.

Time-series genome-wide gene-expression analysis on migrating HAoECs showed that α-MSH treatment significantly affected large gene-sets; this indicates that MC1R activation has a wide influence on pathways playing a prominent role in regulating cellular activity. MC1R activation also modulated genes associated with the endomembrane system and intracellular organelle lumen, suggesting a role in controlling cellular trafficking and molecule mobilization. Remarkably, MC1R engagement with α-MSH modulated the ECM-receptor interaction pathway, which is known to be critical for the directional EC migration (Davis and Senger 2005). Conversely, MC1R activation did not affect the expression of cell cycle-related genes, which was consistent with the apparent lack of regulation of HAoEC proliferation in the gap closure assay. Our findings suggest that the regulation of the ECM components, *i.e.* collagens and laminins, and of their receptors, *i.e.* integrins and dystroglycans, through MC1R may drive higher HAoEC motility. Binding of integrins to type-I collagen suppresses cAMP production and the activity of cAMP-dependent protein kinase A: consequently, actin polymerization is induced, contributing to the formation of stress fibers and to EC contractility, which finally generates the directional movement (Whelan and Senger 2003). This is consistent with the idea that the fine-tuning of integrins and their binding molecules promoted by MC1R activation plays a key role in conditioning HAoEC migration rate. In addition, MC1R stimulation

induced an early upregulation of SNARE proteins (which mediate vesicle-membrane fusion) and cytoplasmic vesicle genes, followed by a later overexpression of metal-binding proteins. Importantly, trafficking and delivery/fusion of vesicle proteins are essential for the regulation of front-rear polarity during directional cell migration (Skalski, Yi et al. 2010; Lizama and Zovein 2013).

Smooth muscle cells express functional melanocortin receptors

We have shown that HAoSMCs express mRNA and proteins of MC1R and MC4R but did not express POMC. It has already been demonstrated that MC1R is expressed on human cerebral (de Angelis, Sahm et al. 1995) and cutaneous (Hartmeyer, Scholzen et al. 1997) microvascular EC and on human umbilical vein ECs (Rinne, Nordlund et al. 2013), but the expression in arterial macrovasculature cells has not been investigated yet. The receptors were functionally active since treatment with α-MSH increased cAMP production in HAoSMC.

Peripheral expression of MC1R is known to be involved in the control of inflammation (Catania, Gatti et al. 2004) and regulate melanocyte, melanoma cell (Zhu, Lalla et al. 2004), lymphocytes (Brod and Hood 2008), and neutrophils (Capsoni, Ongari et al. 2007) migration; expression on macrovascular SMCs suggested local effects on these cell types.

The MC4R expression on HAoSMC, instead, opens new horizons about peripherally receptor functions. MC4R has never been reported in human peripheral tissues: this receptor has been located in the central nervous system and studied for its implication in energy homeostasis and metabolic processes (Tao 2010). Ligand-induced activation of brain MC4R results in inhibition of food intake and stimulation of energy expenditure, promoting a negative energy balance (Balthasar, Dalgaard et al. 2005). In mice, treatment with a specific MC4R agonist controls plasma level of LDL cholesterol by reducing liver PCSK9 expression (Clemmensen, Finan et al. 2015). Therefore, we may speculate that peripherally expressed MC4Rs could be sensors for the metabolic status of the cells.

MCR activation promotes the contractile phenotype and decreases proliferation and migration of the HAoSMCs

Concerning human aortic SMCs, differently from ECs, we observed that α-MSH treatment decreased cell migration. Moreover, it promoted the switch from a synthetic to a contractile

phenotype and reduced cell proliferation. The effects of α-MSH observed on vascular SMC migration is consistent with the multiple evidence that this peptide is able to modulate migration of different cell types, such as melanoma cells (Zhu, Lalla et al. 2004), lymphocytes (Brod and Hood 2008), and neutrophils (Capsoni, Ongari et al. 2007).

Vascular SMC phenotypic switching, migration and proliferation are hallmarks of vascular remodeling, which in turn underlies onset and progression of pathological processes such as neointima hyperplasia, vessel stenosis, and atherosclerosis (Owens, Kumar et al. 2004). Promotion of SMC contractile phenotype by MCR activation is in line with melanocortin anti-inflammatory properties: vascular SMCs, in fact, respond to pro-inflammatory molecules by switching from a contractile, quiescent to a synthetic, proliferative and pro-migratory phenotype (Owens, Kumar et al. 2004; Beamish, He et al. 2010). We showed that in human aortic SMCs the protein markers of the contractile phenotype (α-SMA, SM-MHC) were up-regulated after α-MSH treatment and we showed a concomitant change in cell morphology *in vitro*: the cells were more elongated than the control cells with a higher ratio of axis length.

The phenotypic control of SMCs is a fundamental step to avoid neointima formation in pathological vascular remodeling. *In vitro* studies showed that treatment of rat aortic SMCs with Rosiglitazone, a PPARγ (peroxisome proliferator-activated receptor) agonist, inhibited the switch from contractile to synthetic phenotype. *In vivo* treatment with Rosiglitazone significantly inhibited the neointima formation in rat carotid artery after angioplasty (Yang, Kim et al. 2013). Rapamycin, used in drug-eluting stents, inhibited *in vivo* intima hyperplasia in both human and animal model; *in vitro*, Rapamycin promotes vascular SMC contractile phenotype (Martin, Merenick et al. 2007). Opposite to the above-mentioned compound, α-MSH is an endogenous peptide with potent anti-inflammatory proprieties (Catania, Lonati et al. 2010) able to maintain the vascular homeostatic state: given the absence of side effects in preclinical studies on inflammatory diseases, it could be proposed as a better therapy to control remodeling and neointima formation. In parallel with the phenotype switch, α-MSH reduced HAoSMC migration, and proliferation. We showed that HAoSMC migration decreased in response to α-MSH even in presence of a chemoattractant molecule such as PDGF-BB. Previous studies showed the involvement of the melanocortin system in the regulation of cell migration and proliferation, in particular of inflammatory cells: α-MSH reduced secretion of IL-8 e Gro-α, pro-inflammatory cytokines and halted monocyte migration through inflammatory sites (Genedani, Bernardi et al. 1990). Moreover, α-MSH reduced T-lymphocytes

(Cooper, Robinson et al. 2005), neutrophils (Capsoni, Ongari et al. 2007) and eosinophils (Catania, Gatti et al. 2004) migration. α-MSH regulates migration and proliferation of keratinocytes in inflammatory conditions (Schauer, Trautinger et al. 1994) and exerts anti-proliferative and anti-migratory actions on melanoma cells (Zhu, Lalla et al. 2004). In other cases, opposite to our findings, α-MSH seems to have pro-proliferative properties and mitogenic effects, in particular on melanocytes (Suzuki, Cone et al. 1996), but this effect is linked with one of its primary homeostatic functions, *i.e.* melanogenesis and protection from UV.

The activation of MCRs locally expressed on HAoSMC downregulate cluster of genes implicated in cell migration and proliferation

Molecular profiling of human cells/tissues represents an effective approach to unveiling the "fingerprint" of any changes occurring in response to environmental challenges, different healthy/disease state, or specific genetic/epigenetic programs. Therefore, to corroborate the effects we observed on SMC migration and proliferation, we investigated the transcriptome of α-MSH-stimulated SMCs to associate modulated gene expression patterns with specific biological functions. We focused our analysis on samples collected at hour 6 of the time-course migration assay, since, at this time point, we expected to find the main gene expression changes explaining the larger migration differences observed at 9 hours between α-MSH-stimulated vs. non-stimulated SMC.

Gene set enrichment analysis revealed that the treatment of SMC with α-MSH was strongly associated with a lower expression of genes implicated in cellular migration and proliferation, wound healing and ECM deposition. Consistently, these findings fit with the results obtained in a gap-closure migration *in vitro* assay on α-MSH-stimulated SMC, thus supporting a role for MC1R activation in the down-regulation of vascular SMC migration and proliferation.

To identify specific transcriptional programs that might help to reconstruct the mechanism of action that would lead to a decreased SMC migration/proliferation through MC1R activation, we performed a gene set enrichment analysis using transcription factor targets gene sets. This approach would have allowed us to identify transcription factors that could likely associate with significant gene sets in either α-MSH-stimulated SMC or control cells. We found that the

transcription factors HSF1, SOX9, and NKX2.5 were significantly associated with down-regulated genes in α-MSH-stimulated SMC. Notably, these transcription factors are known to control the expression of genes implicated in cellular migration (Salinthone, Tyagi et al. 2008), vascular SMC differentiation (Xu, Ji et al. 2012), and regulation of Collagen Type I (Ponticos, Partridge et al. 2004), respectively, suggesting that the phenotypic changes we observed on SMC after α-MSH stimulation are orchestrated by precise signaling pathways. In this context, we hypothesized that the possible mechanism that specifically links MC1R activation and SMC migration would act through the down-regulation of and/or interference in the HSF1 signaling.

HAoSMC migration is modulated by the downregulation of the P38MAPK/HSF1 pathway

HSF1 controls the expression of heat shock proteins (HSPs) and previous work established that increased expression of HSPs in the vessel wall might evoke proinflammatory and autoimmune reactions in the pathogenesis of atherosclerosis (Berberian, Myers et al. 1990; Zhu, Quyyumi et al. 2001). Moreover, HSF1 was highly expressed as well as activated in atherosclerotic lesions of hypercholesterolemic rabbit model (Metzler, Abia et al. 2003) and mouse HSF1 deficiency alters cardiac redox homeostasis and increases mitochondrial oxidative damage (Yan, Christians et al. 2002). HSF1 is a substrate for p38MAPK (Dayalan Naidu, Sutherland et al. 2016) and it is known that p38MAPK activation drives cell migration in vascular SMC (Hedges, Dechert et al. 1999; Kavurma and Khachigian 2003). p38MAPK is activated by mitogenic and migratory stimuli, such as by pro-inflammatory cytokines of immune-cells under stress condition (Coulthard, White et al. 2009; Rose, Force et al. 2010). Some studies demonstrated that p38MAPK pathway inhibition in pathologic condition prevented neointima formation. In vitro inhibition of p38MAPK phosphorylation blocked vascular SMC polarization in response to mechanical stress (Chen, Qin et al. 2003). In a rabbit *in vivo* model of artery injury, chemical inhibition of p38MAPK slowed down vascular remodeling (Ju, Nerurkar et al. 2002). Moreover, in mice, p38MAPK pathway inhibition halted vascular SMC proliferation, DNA replication and Rb protein phosphorylation (Proctor, Jin et al. 2008). It is also known that p38MAPK is a key element in the regulation of pro-inflammatory molecule transcription and translation. Substrates downstream the pathway of p38MAPK, such as TNF-α, IL-1 and the

HSP, are among the main factors involved in atherosclerotic progression (Spagnoli, Bonanno et al. 2007).

We showed here, for the first time, that the p38MAPK pathway is de-activated in response to the stimulation of MC1R in macrovascular SMC. Moreover, we showed that HSF1 expression is downregulated and we propose that the negative modulation of the p38MAPK/HSF1 pathway is the mechanism of action underlying MC1R control on SMC migration. As mentioned above, p38MAPK is a key player in vascular remodeling and different studies in the last 20 years focus on the inhibition of this pathway searching for a system to avoid systemic effects (Coulthard, White et al. 2009; Church, Martin et al. 2015). Melanocortin receptor expressed on macrovascular cells could be an effective target to modulate the p38MAPK pathway locally and consequently control maladaptive vascular remodeling.

Limitations

The main limitation of our study is that we do not have an *in vivo* model to confirm the role of MCR activation in remodeling and neointima formation directly on injured arteries. We might perform future experiments on conditional knockout mice for MC1R and MC4R in SMC and EC.

Relevance and conclusions

Consistent with the known peripheral effects of MC1R activation (Zhu, Lalla et al. 2004; Capsoni, Ongari et al. 2007; Brod and Hood 2008), we showed for the first time that its activation with α-MSH controls human aortic EC and SMC migration. Intriguingly, the effects on migration are different in the two cell types. Our data revealed that MC1R activation by α-MSH increased the rate of HAoEC migration in a wound-healing gap-closure assay, without significantly affecting cell proliferation. Opposite, α-MSH decreased the rate of HAoSMC migration, both in a gap-closure and in transwell-chemotactic assays. This effect was specifically induced by MC1R activation, since the MC1R antagonist, but not the MC4R antagonist, was able to revert the migration rate to the same of the control cells. These data suggest strictly cell-related peripheral effects of α-MSH on the macrovascular cells, which upon injury or pro-inflammatory stimuli could regulate cells behavior locally promoting

endothelial repair and avoiding neointima formation, thus contrasting the hallmark events in vascular remodeling (Mulvany, Baumbach et al. 1996; Owens, Kumar et al. 2004).

Considering our results, it could be interesting to test exogenous ligands of melanocortin receptors as potential drugs to modulate vessel function/dysfunction in a pathological state. The small size of α-MSH makes it an attractive molecule for drug design. Melanocortin analogs, such as Melanotan I and II have already been used in human as tanning agents to increase eumelanin production to protect against UV-induced DNA damage (Hadley ME, Dorr RT., 2006). Importantly, recent studies propose the use of α-MSH analogs to treat skin cancer and melanoma formation (Raposinho PD et al., 2008; Quinn et al., 2010). There is also a great interest in MC4R as target for the design of novel therapeutics to treat disorders of body weight, such as obesity and cachexia (Fani L. et al., 2014). We may suppose a positive effects of the administration of α-MSH analogs to control the inflammatory state in vessel pathologies such as atherosclerosis or re-stenosis processes. Giving that the MCS contributes to the restoration of homeostasis in an perturbed system, melanocortin administration could modulate the main aspects of pathological vascular remodeling, promoting endothelial repair and SMC switching to the contractile form. Finally, since systemic administration of α-MSH analogs may control a wide spectrum of body functions, we envisaged applications as locally delivered drugs.

For instance, a possible clinical application of our studies should be the development of new drug-eluting stents with local deliver of melanocortin peptides to antagonize re-stenotic processes without affecting EC proliferation and wound closure. Drug-eluting stents (DES) have excellent outcomes over the short to medium term, but stent thrombosis and in-stent restenosis are possible adverse consequences of stent implantation. Second-generation stents are coated with more specific, more effective and selective anti-proliferative agents, which have lower systemic toxicity and do not delay endothelial healing such as Everolimus and Zotarolimus. Last studies focused on strategies that aim at re-endothelialization of the stent struts combined with effective approaches to prevent smooth muscle cell proliferation and migration (Puranik, Dawson et al. 2013; Byrne, Joner et al. 2015): in this contest, MCR activation by melanocortins could be an interesting target. Since the melanocortin system is an endogenous system with potent anti-inflammatory proprieties (Catania, Lonati et al. 2010) and able to maintain the vascular homeostatic state, targeting MC1R could be an alternative and promising strategy to treat *in loco* the endothelial lesion or dysfunction and to prevent

SMC phenotypic switch, proliferation, and migration caused by pathologic remodeling or stent implantation.

In conclusion, our results provide new knowledge about MCS involvement in vessel homeostasis and, possibly, on melanocortin modulation of vascular inflammation and remodeling. The use of anti-inflammatory molecules such as α-MSH could represent a strategy to prevent and heal endothelial dysfunction in macrovascular arteries and to maintain SMCs in the differentiated state or to revert their phenotype from a proliferative state. MC1R could represent a target for new therapeutic strategies aimed at preventing/repairing endothelial injury and neointima formation in a variety of cardiovascular pathological conditions.

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PUBLICATIONS

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