Experimental Cell Research 392 (2020) 111997

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

The microproteome of cancer: From invisibility to relevance

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ARTICLE INFO

Keywords:
Microprotein
Micropeptide
SEPs
sORF
smORF
alt-ORF
Cancer
Tumor suppressors

ABSTRACT

Recent findings have revealed that many genomic regions previously annotated as non-protein coding actually contain small open reading frames, smaller that 300 bp, that are transcribed and translated into evolutionary conserved microproteins. To date, only a small subset of them have been functionally characterized, but they play key functions in fundamental processes such as DNA repair, RNA processing and metabolism regulation. This emergent field seems to hide a new category of molecular regulators with clinical potential. In this review, we focus on its relevance for cancer. Following Hanahan and Weinberg's classification of the hallmarks of cancer, we provide an overview of those microproteins known to be implicated in cancer or those that, based on their function, are likely to play a role in cancer. The resulting picture is that while we are at the very early times of this field, it holds the promise to provide crucial information to understand cancer biology.

1. Introduction

Recent advances coming from computational analyses, peptidomics and ribosome profiling have revealed that our proteome includes a new class of small proteins produced by the translation of small open reading frames shorter than 300 bp in length, generating proteins that have been called microproteins (also known as micropeptides or SEPs, from small ORF-encoded peptides) [1,2]. The main reason why microproteins have been overlooked till recently is that most ORF-prediction algorithms -including the one used by FANTOM annotation consortium- placed an arbitrary cut off of 300 bp, missing the proteins below 100 amino acids [3,4]. Although nomenclature has been inconsistent in the field, based on their location, the ORFs encoding microproteins can been classified as 1) small ORFs, “sORFs”, when they are located in assumed non-coding transcripts such as lncRNAs, miRNAs and circRNAs, or 2) “alt-ORFs”, when they are inside annotated coding genes starting from alternative start codons. Alt-ORFs can be located in the UTRs (typically called "uORFs" when they are in the 5′UTR) or overlapping the reference coding sequence. In this review, we focus on the microproteins derived from sORFs. Although only a subset of them have been functionally characterized, growing evidences demonstrate that microproteins are indeed active proteins playing important functions in a plethora of processes including RNA processing, DNA repair, metabolism regulation and regeneration [5–9]. Microproteins might be particularly well suited to fine-tune complex processes as regulation of enzyme activity, intracellular signal transduction and cell surface signaling, but extensive research in the field is needed to decipher additional functions of the microproteome [1]. These findings open a new category of molecular regulators with implications from basic research to the clinical setting.

Cancer is a complex and multistep disease in which normal cells, through the succession of several genetic and epigenetic events acquire the capacity to grow, invade adjacent tissues, disseminate and ultimately colonize distant organs. Although the specific mechanisms that allow neoplastic transformation and metastasis may vary between different cancer types, there are common regulatory circuits that collectively govern carcinogenesis. In 2000, Hanahan and Weinberg published a seminal paper in which they postulated six capabilities shared by most human tumors [10], which was revisited in 2011 to finally include eight hallmarks of cancer: sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism and the evasion of immune destruction (Fig. 1) [10]. Moreover, they proposed two enabling characteristics that represent the mechanisms by which the hallmarks of cancer are acquired: genome instability and the inflammatory state of premalignant lesions (Fig. 1). Although in the last decades we have witnessed a great advance in molecular oncology, we are still far from fully understanding how the hallmarks of cancer are acquired and maintained to sustain tumors. The new information emerging from the study of microproteins suggests that they constitute an important source of cancer regulators implicated in multiple hallmarks of cancer.

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https://doi.org/10.1016/j.yexcr.2020.111997
Received 8 January 2020; Received in revised form 2 April 2020; Accepted 3 April 2020
Available online 14 April 2020

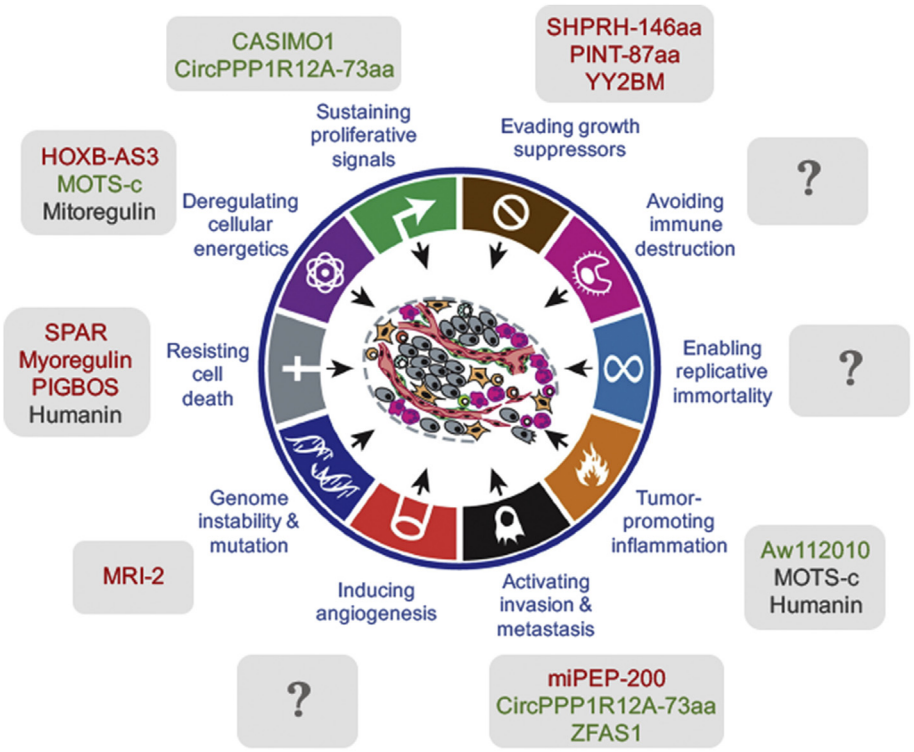

Fig. 1. Microproteins as novel regulators of the hallmarks of cancer. A subset of microproteins have been functionally characterized and have been directly related with cancer; some others, based on their function, are likely to be related with cancer. The figure represents the hallmarks of cancer defined by Hanahan and Weinberg and their related microproteins. In green, the microproteins that promotes or activate the hallmark and, in red, the microproteins that function inhibiting or blocking the hallmark. The ones in grey need further investigation to be classified. Of interest, those depicted in red represent tumor suppressor microproteins with potential pharmacological activity, while those in green are pro-oncogenic peptides that could be targeted in the clinic. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

1.1. Sustaining proliferation signals

In physiologic conditions, mitogenic signals are strictly controlled to ensure the maintenance of normal tissue architecture and homeostasis. By contrast, cancer cells are mitogenically overstimulated. Such mitogenic hyperactivity can be achieved in several ways: First, neoplastic cells can produce their own growth-promoting signals in an autocrine manner or, alternatively, produce paracrine factors that stimulate the release of mitogens by neighbor stromal cells. Second, specific somatic mutations can trigger constitutive activation of growth factor receptors or its downstream components, converting those elements of the signaling cascades in "*bona fide* oncogenes". Recent studies have shown the importance of some microproteins regulating mitogenic signaling. The first sORF described with oncogenic activity has been Cancer-Associated Small Integral Membrane Open reading frame 1 (CASIMO1). CASIMO1 is expressed in hormone-dependent breast cancer during all stages of malignancy, and its deficiency reduces cell proliferation in several breast cancer cell lines [11]. CASIMO1 interacts with squalene epoxidase (SQLE), an oncogene that promotes ERK phosphorylation and MAPK pathway activation [12]. Remarkably, CASIMO1 deficiency reduces ERK phosphorylation while exogenous overexpression of SQLE is sufficient to rescue the loss of CASIMO1, suggesting that CASIMO1 might be modulating ERK activation via SQLE interaction [11]. These observations are in line with a role of CASIMO1 as an oncogene, acting as a positive regulator of MAPK cascade in breast cancer cells. More recently, another microprotein has been proposed to act as a positive regulator of the Hippo-Yap pathway in colon cancer. CircPPP1R12A-73aa is a microprotein encoded by CircPPP1R12A, the most abundant circular RNA (circRNA) in colon cancer, and its overexpression leads to increased cell proliferation [13]. CircPPP1R12A-73aa induces the transcriptional upregulation of Hippo-Yap pathway components and increases YAP1 protein levels, suggesting a possible role of CircPPP1R12A-73aa as an activator of the Hippo pathway [13]. Although CircPPP1R12A-73aa's mechanism of action needs to be further studied, together with CASIMO1 exemplify the relevance of sORFs regulating mitogenic signals, and how they can be exploited by cancer cells to sustain their proliferation needs.

1.2. Evading growth suppressors

In homeostasis, powerful signaling programs block the proliferation of damaged or potentially malignant cells. Signals that activate growth suppression are integrated by the cell in a highly complex manner to decide whether to halt cell-cycle progression, activate apoptotic programs or enter senescence [14,15]. These signaling programs are mainly governed by tumor suppressor proteins and, for neoplastic transformation to occur, these tumor-suppressing mechanisms need to be inactivated [16–18]. Thus, a good understanding of tumor suppression pathways is crucial and might reveal novel therapeutic options in cancer. In this regard, the sORF-encoded proteome can provide new insights on the biology of tumor suppression mechanisms and could represent a novel source of therapeutic agents. Several microproteins have already been shown to play a role in regulating tumor suppression mechanisms through different ways. The *LINC-PINT* gene was already reported to produce a lncRNA regulated by p53 [19], but in its circular form, it contains a sORF that encodes an 87-amino acid microprotein. This microprotein, PINT-87aa, suppresses tumorigenic capabilities of glioblastoma cell lines *in vitro* including cell proliferation, self-renewal and anchorage independent growth, and its deficiency results in increased tumor burden *in vivo*. The authors showed that this microprotein interacts with the polymerase associated factor (PAF1) complex, essential for RNA II polymerase binding and transcription elongation [20]. The interaction of PINT-87aa with PAF1 pauses RNA II polymerase at specific oncogene promoters -such as Cyclin D1, CPEB1, c-MYC and SOX2-impairing their transcription [21].

Although it is bigger than 100 amino acids, and therefore strictly not generated from a sORF, another interesting example of the coding potential of circ-RNAs is SHPRH-146aa. SHPRH is an E3-ubiquitin ligase that promotes the degradation of PCNA (Proliferating Cell Nuclear Antigen), impeding cell cycle progression through S-phase. Recently, Zhang and colleagues have revealed a circular RNA derived from the *SHPRH* primary transcript that codes for SHPRH-146aa. SHPRH-146aa stabilizes SHRPH by preventing its degradation, and thereby promoting the ubiquitination of PCNA. Accordingly, the overexpression of SHPRH-146aa reduces the proliferation of glioma cell lines *in vitro* and *in vivo*

[22]. Importantly, both microproteins, SHPRH-146aa and PINT-87aa are silenced or downregulated in glioblastoma [19][22], further supporting their tumor suppressor potential and opening the possibility of using these small proteins as therapeutic agents.

More recently, the Y-chromosome-linked lncRNA LINC00278 has been shown to encode YY1BM, a microprotein with tumor suppressor activity in esophageal squamous cell carcinoma (ESCC). This microprotein induces apoptosis through the androgen receptor pathway under nutrient deprivation. Interestingly, YY1BM is downregulated by cigarette smoking in human males with ESCC, increasing the survival of cancer cells under nutrient deprivation. Moreover, intratumoral injection of the purified microprotein showed a therapeutic effect in xenograft models, suggesting its potential as a tumor suppressor agent [23].

Last, it is worth to mention that some identified microproteins, like NoBody, have not been directly linked with cancer but they regulate fundamental processes that can impact on cancer cells, such as mRNA decay [5]. Nonsense Mediated Decay is a complex process that can be exploited by cancer cells to degrade the mRNA of tumor suppressor genes. On the other hand, it can also be used as a therapeutic intervention to target oncogene-encoding mRNAs [24]. Thus, the role of this microprotein (and many others) as an oncogene or as a tumor suppressor might be highly tumor specific and dependent on the cellular context.

1.3. Resisting cell death

Regulation of the balance between cell death and survival is critical for maintaining tissue homeostasis. The induction of programmed cell death by apoptosis is a natural barrier for neoplastic transformation [25]. Signals that activate apoptosis are sensed and integrated, among others, by the pro- and anti-apoptotic proteins of the Bcl-2 family. Anti-apoptotic members of the family (Bcl-2, Bcl-x_L, Bcl-w, Mcl1, A1) interact with pro-apoptotic proteins Bax and Bak, suppressing their function. Upon certain pro-apoptotic stimuli, this interaction is broken allowing Bax and Bak to disrupt mitochondrial membrane, which releases cytochrome c to the cytosol, activating in turn the caspases cascade that ultimately disassembles the cell [26]. Interestingly, certain sORFs appear to be important fine-tuning regulators of this process. The microprotein Humanin (HN) is encoded by a sORF in the mitochondrial 16s ribosomal RNA gene, although a nuclear origin of this microprotein has not been ruled out yet due to the presence of similar ORFs in the nuclear genome [27]. HN was first described as a neuroprotective agent in Alzheimer disease [28]. Further studies have shown that its cytoprotective activity is, at least in part, due to its ability to block apoptosis through direct interaction with Bax [29] or by binding and inhibiting the Bax activator BimEL [30]. Regarding cancer, HN is expressed in gastric and bladder cancer and induces chemotherapy resistance [31]. On the other hand, the cytoprotective activity of HN could be beneficial for normal tissues, given that HN also reduces the side-effects of chemotherapy in non-cancer cells [31]. Further studies are needed to clarify the potential role of HN in the clinical setting.

Together with apoptotic suppression, cancer cells activate cell survival mechanisms to avoid cell death. mTOR is a serine/threonine kinase that integrates multiple environmental cues, and it is activated to promote cell growth and survival in favorable conditions [32]. Hyperactivation of mTOR has been reported in more than 70% of cancers [33] and, therefore, mTOR inhibition is an approach currently used in anti-cancer therapies. SPAR is a lncRNA-encoded microprotein that inhibits mTORC1 by its interaction with the lysosomal v-ATPase. Depletion of SPAR *in vivo* has been shown to improve muscle regeneration through higher mTORC1 activity [34]. Among the multiple mechanisms that regulate mTORC1, SPAR appears to reduce the amino-acid sensing route of mTORC1 activation. Although it has not been tested, SPAR expression could have anti-tumor properties in specific cancer types where mTORC1 activation occurs through amino-acid sensing pathways, as it is the case of some lymphomas [35].

On the other hand, the activation of the cellular stress response is an essential mechanism that helps healthy cells to cope with stress and damage, and it is co-opted by malignant cells to thrive in highly adverse conditions and avoid cell death. Accordingly, Unfolded Protein Response (UPR) and endoplasmic reticulum (ER)-stress response have been reported to be upregulated in many cancers, helping them to deal with high protein synthesis demands while protecting them from stress-induced cell death [36,37]. These pro-survival responses are highly dependent on cytosolic Ca^{2+} concentration and the ER is the main responsible of intracellular Ca^{2+} storage [38]. MYOREGULIN (MLN), encoded by a previously annotated long non-coding RNA works as an inhibitor of the Ca^{2+} ATPase pump SERCA [39]. Inhibition of SERCA activity increases cytosolic Ca^{2+} concentration, sensitizing cells to cell death. For this reason, SERCA inhibition is being tested as a potential therapy in tumors with hyperactivation of Ca^{2+} channeling activity [40]. Even though MLN has not been related with cancer so far and in homeostasis its expression is restricted to skeletal muscle, several SERCA-inhibitory microproteins have been reported to control Ca^{2+} signaling in a tissue specific manner [41] and their expression might be useful to tackle cancers with high SERCA activity.

Finally, PIGBOS is a novel microprotein encoded by an antisense of the *PIGB* gene. This microprotein localizes in the mitochondrial outer membrane interacting with the ER through the CLCC1 protein. Downregulation of PIGBOS induces apoptosis by increasing sensitivity to chemically-induced UPR [42]. Whereas the molecular mechanism of PIGBOS function has not been described yet, it is reasonable to think that its inhibition in cancer cells may have therapeutic potential by sensitizing cells to UPR and forcing them to enter apoptosis.

1.4. Activation of invasion and metastasis

Carcinomas are tumors that arise from epithelial tissues. The progression from localized tumor to invasive carcinoma and distal metastasis requires changes in cell morphology and in cell-cell and cell-extracellular matrix (ECM) attachment. The “epithelial-to-mesenchymal transition” (EMT) is a cellular program involved in embryonic morphogenesis and wound healing. Cancer cells can also activate the EMT program to acquire the invasive phenotype needed for metastatic spread [43]. Biological traits acquired during EMT include the loss of adherent junctions, the acquisition of spindle/fibroblastic morphology, the expression of matrix-degradation enzymes, increased motility and resistance to apoptosis. Importantly, while EMT is needed for metastasis, it must be reversed to colonize a new organ through a process known as “mesenchymal-to-epithelial transition” (MET) [43]. Of note, it has been recently proposed that cancer cells may acquire a “plastic-hybrid state” or a “partial EMT” state. According to this vision, cancer cells acquire mesenchymal features while continuing to express epithelial traits, resulting in a selective advantage during the metastatic process [44].

Many non-coding RNAs have been shown to regulate EMT, highlighting the importance of miR-200a and miR-200b as key negative EMT regulators, which are usually epigenetically repressed in cancer cells [45]. Of note, a recent study has identified two potential microproteins encoded by the miR-200a and miR-200b pri-miRNAs, the precursor transcripts of the miRNAs, which have been named miPEP-200a and miPEP-200b. The expression of these sORFs seems to be associated with a decreased migration in wound healing assay and with diminished expression of the mesenchymal marker Vimentin [46]. More recently, the lncRNA ZFAS1 has been shown to translate a microprotein that is proposed to promote cell migration by elevating intracellular reactive oxygen species (ROS) production [47]. Additionally, the microprotein CircPPP1R12A-73aa (described above) increases cancer cell migration and invasion *in vitro* and metastasis development *in vivo* [13], possibly reflecting the activation of EMT by the Hippo-Yap pathway [48,49].

Finally, recent evidences suggest that cell-to-cell fusion events,

especially between cancer cells and immune cells, could contribute to the acquisition of metastatic behaviors [50]. Importantly, microproteins can regulate cell fusion, as it has been shown for MYOMIXER, an 84-amino acid peptide necessary for heterotypic fibroblast-myoblast fusion [51]. Although speculative, it is possible that there are non-identified microproteins playing important functions in metastasis by regulating cell fusion. Although more investigations are needed to have a complete picture, all together these discoveries point to the microproteome as a source of regulators of cancer invasion and metastasis.

1.5. Deregulating cellular energetics

Another important feature of cancer cells is that they reprogram their metabolism in different ways to comply with their highly demanding energetic needs. One of these strategies is to rely on glycolysis rather than on oxidative phosphorylation (OXPHOS) as their primary energy production mechanism, even in the presence of oxygen [52]. The process of metabolic reprogramming results from a complex regulation between mitochondrial genes, tumor suppressors and oncogenic pathways and its targeting is currently being tested as a therapeutic strategy [53]. Importantly, microproteins have emerged as important regulators of this process with potential clinical implications. The lncRNA HOXB-AS3 has been shown to produce a 53-amino acid microprotein involved in RNA splicing. Huang and collaborators showed that HOXB-AS3 is downregulated in colorectal cancer cells, which changes the splicing of Pyruvate Kinase M (PKM) pre-mRNA to re-express the embryonic isoform PKM2, that favors glycolytic activity. By contrast, expression of HOXB-AS3 peptide favors the expression of the adult isoform (PKM1), that promotes oxidative phosphorylation. Collectively, they demonstrate that overexpression of HOXB-AS3 peptide in colorectal cancer cell lines attenuates their oncogenic capacity by altering their use of glucose metabolism [6]. Other microproteins have also been described to play a role in metabolic regulation, although their role in cancer is yet to be investigated. Specifically, MITOREGULIN (also called MOXI or MPM) is a 56-amino acid microprotein encoded by LINC00116, a muscle-enriched lncRNA. The function of this protein has been proposed to rely on its interaction with different inner mitochondrial proteins, increasing respiratory efficiency through the stabilization of supercomplexes in the electron transport chain [54,55] and promoting long-chain fatty acid ß-oxidation [56]. It would be of interest to study the role of this microprotein in cancer cell metabolism. Finally, the mitochondrial genome also plays a role in metabolic activity and, despite its small size, it has been described to contain several sORFs, like the one encoding for MOTS-c (mitochondrial open reading frame of the 12*s* rRNA-c). *In vitro*, MOTS-c increases glucose uptake, glycolytic activity and AMPK activation, while reducing oxygen consumption rate. Accordingly, MOTS-c improves metabolic parameters associated with obesity *in vivo* [57]. Given that MOTS-c favors a glycolytic program and activates AMPK, it might be beneficial for cancer cells, which would be interesting to be addressed in the future.

1.6. Enabling characteristic: genomic instability and mutations

As proposed by Hanahan and Weinberg [10], accumulation of genomic alterations during carcinogenesis is an event that enables the acquisition of the core hallmarks discussed above. Tumor progression can be seen as a succession of clonal expansions: mutations are randomly accumulated in the pool of cancer cells and, eventually, advantaged genotypes enabling cell survival and growth are selected under the pressure of environmental stimuli. Due to the adaptive advantage that a high mutational rate confers to cancer cells, the components of the genomic maintenance machinery are often affected in neoplasia. Typically, the accumulation of mutations can be accelerated by defects in the sensors of DNA damage, in components of DNA repair machinery and/or in effectors that force damaged cells to enter senescence or apoptosis [58]. Thus, microproteins involved in the maintenance of genome stability may behave as tumor suppressor genes and are likely to be affected in cancer cells. Slavoff and collaborators identified MRI-2 as a 69-amino acid microprotein coded by *C7orf49* gene, also known as “modulator of retrovirus infection homolog” (MRI) [8]. MRI-2 interactome analysis revealed that this microprotein interacts with Ku70 and Ku80 proteins, the two subunits of the heterodimeric protein Ku, key effector of the non-homologous-end-joining (NHEJ) pathway for DNA double-strand break (DSB) repair. When a DNA DSB occurs, the first protein that binds to the break is the Ku heterodimer, which allows the recruitment of the DNA-dependent protein kinase (DNA-PK) and additional factors which in turn repair the DSB [59]. MRI-2 is accumulated in the nuclei of DBS-induced cells, and recombinant MRI-2 increases NHEJ *in vitro* [8]. The mechanism by which MRI-2 enhances NHEJ has not been fully addressed, but it is possible that the interaction of MRI-2 with Ku proteins may improve their DNA-binding affinity or facilitate the recruitment of other repair complex components. Although in this review we focus in sORF-derived microproteins, it is worth to mention that many alt-ORFs and uORFs can produce functional microproteins with potential roles in genome instability. For example, the AltMRVI1 microprotein is coded by an alt-ORF inside the MRVI gene, and it directly interacts with BRCA1, one of the key effectors of homologous recombination DNA repair machinery [60,61]. The discovery of microproteins involved in genomic stability maintenance suggests that there could be numerous “genomic regulator-microproteins” which help preventing accumulation of genomic alterations in cancer cells.

1.7. Enabling characteristic: tumor-promoting inflammation

Another characteristic that allows the acquisition of the core cancer hallmarks is inflammation [10]. Several microproteins already described in this review might be of particular interest regarding tumor inflammation. In particular, the MDPs MOTS-c and HN have demonstrated their capacity to exacerbate the pro-inflammatory effect of senescence-associated secretory phenotype (SASP) of senescent cells by modulating their mitochondrial activity [62]. Cellular senescence is activated by multiple cellular stressors and it is characterized by a stable cell-cycle arrest and a pro-inflammatory secretome. Senescence acts as a main tumor suppressor barrier that impedes neoplastic transformation of damaged cells and promotes tissue repair [17]. However, the accumulation of senescent cells in tissues could have detrimental effects, mainly because of the inflammatory SASP and, in tumors, the presence of senescent cells promotes cancer cell growth and metastasis [63,64]. In this regard, it would be interesting to study whether the pro-inflammatory cytokines upregulated by MDPs facilitate the immunoclearance of senescent tumor cells or, on the contrary, favor a pro-tumorigenic microenvironment. In addition, ribosome profiling of bone marrow-derived macrophages revealed Aw112010, a non-ATG-initiated microprotein that promotes a pro-inflammatory response increasing canonical inflammatory cytokines like IL-6 and IL-12p40 upon bacterial infection [65]. These findings suggest a role of microproteins in cancer development through regulating inflammation.

2. Concluding remarks

sORF-encoded proteins have expanded our view about the coding potential of the genome, adding a new layer of complexity in the regulation of biological processes. The emergent picture suggests that microproteins allow the fine-tuning of many of these processes to adapt to specific needs and cellular contexts. Here, we have summarized what might be their implication in cancer. Even if only a small subset of microproteins have been functionally characterized so far, there is evidence of many of them as regulators of most of the hallmarks of cancer and its enabling characteristics (Fig. 1). While, so far, microproteins have not been directly related to angiogenesis, replicative immortality and immune evasion, we should take into account that the

proposed hallmarks are interconnected and some of the already identified microproteins, upon further analysis, could be classified in several hallmarks at the same time.

Finally, it is worth mentioning that most sequencing efforts in cancer restrict their analysis to the annotated protein-coding genome, unintentionally ignoring the microproteome. Therefore, it remains unclear if mutations in microproteins are selected during cancer evolution. If this were the case, mutated microproteins could also be a source of cancer neoantigens that can be used to improve the development of personalized immunotherapy [66]. We envision that this is an area that is going to be intensively studied and expanded in the coming years, and will bring crucial information for the clinic.

Here, we have discussed a set of microproteins encoded by lncRNAs, miRNAs, rRNAs, and cirRNAs but many more are yet to be explored, including the ones coded by the so-called alt-ORFs, that we have not addressed in this review. We are at the beginning of a new set of discoveries in which the identification and characterization of the cellular microproteins repertoire -the microproteome- will help us to better understand how physiological and pathological processes are regulated at its finest level. We anticipate that advances in this field will bring new therapeutic opportunities for oncology.

CRediT authorship contribution statement

Iñaki Merino-Valverde: Conceptualization, Writing - original draft, Writing - review & editing. **Emanuela Greco:** Conceptualization, Writing - original draft, Writing - review & editing. **María Abad:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Acknowledgements

M.A. is a recipient of a Ramón y Cajal contract from the Spanish Ministry of Economy (MINECO). I.M. is a recipient of an FPI contract from the Spanish Ministry of Economy (MINECO). Work in the laboratory of M.A. is funded by Fero Foundation and by grants from the Spanish Ministry of Economy (RTI2018-102046-B-I00), from *"la Caixa"* Foundation (HR18-00256), and from Mutua Madrileña Foundation.

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