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Advisor: Pedro Augusto Carlos Magno Fernandes, PhD

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To my family and friends.

*“Life isn't as serious as the mind
makes it out to be.”*

Eckhart Tolle
(A New Earth: Awakening
to Your Life's Purpose)

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Resumo

A plasticidade e heterogeneidade das células malignas têm papel fundamental sobre a progressão tumoral e o desenvolvimento de resistência terapêutica. Nos últimos anos, com o advento de tecnologias de sequenciamento de células individuais (scRNA-seq), estudos com diferentes tipos de câncer revelaram que um único tumor pode conter subpopulações de células malignas com perfis transcricionais distintos. Com isso, tornou-se necessário estabelecer modelos experimentais que permitam o estudo da relevância biológica e clínica dessa diversidade celular, bem como os mecanismos moleculares subjacentes. Nesse sentido, geramos dados de scRNA-seq para 198 linhagens celulares (22 tipos de câncer) e caracterizamos sistematicamente a heterogeneidade da expressão gênica. Descobrimos que a coexistência de subpopulações altamente distintas em uma mesma linhagem celular é rara. Por outro lado, padrões contínuos de heterogeneidade de expressão gênica, representados por espectros de estados celulares, são comuns, ocorrem em diferentes linhagens, estão associados a múltiplos processos biológicos e são geralmente independentes da diversidade genética. Notavelmente, apesar de condições *in vitro* não possuírem um microambiente nativo e espacialmente variável, muitos dos padrões contínuos de heterogeneidade observados nas linhagens recapitulam aqueles encontrados em amostras tumorais clínicas, indicando que a plasticidade epigenética intrínseca tem papel fundamental na geração da heterogeneidade intratumoral. Os dados gerados também nos permitiram identificar as linhagens mais adequadas para o estudo da plasticidade celular. Como exemplo, selecionamos duas dessas linhagens modelos e demonstramos a dinâmica temporal e relevância terapêutica de um programa de heterogeneidade recorrente associado à senescência epitelial. Além disso, considerando os meios pelos quais as células malignas se comunicam, isto é, via interações físicas célula-a-célula e secreção de fatores solúveis, também buscamos explorar novas redes de sinalização autócrina/parácrina que possam ser exploradas clinicamente. A melatonina, mais conhecida como "hormônio da pineal", é uma molécula pleiotrópica produzida em diversos tecidos e que vem sendo cada vez mais reconhecida como um agente antitumoral. A melatonina atua por meio de vários mecanismos biológicos, incluindo a eliminação direta de radicais livres e a ativação de receptores de alta afinidade acoplados à proteína G (MT1 e MT2). Na última década, nosso grupo forneceu diversas evidências de que o ajuste fino da produção extrapineal de melatonina durante a inflamação aguda é crítico para a manutenção da homeostase tecidual. Entretanto, nosso conhecimento sobre o papel fisiopatológico da melatonina local em processos malignos é muito limitado. Interessantemente, demonstramos que, em gliomas, a capacidade das células de sintetizar e acumular melatonina correlaciona-se negativamente com o grau malignidade tumoral. Utilizando dados de expressão gênica, desenvolvemos um modelo preditivo do conteúdo de melatonina no microambiente tumoral, o índice ASMT:CYP1B1, o qual se mostrou um fator prognóstico positivo, independente do grau e subtipo histológico dos gliomas. Por fim, buscamos fornecer suporte adicional para o uso racional da melatonina e análogos no tratamento de cânceres cerebrais. Revelamos que em gliomas e meduloblastomas os receptores de melatonina MT1 e MT2 desempenham papéis opostos sobre o controle da progressão tumoral. Notavelmente, compostos que simultaneamente ativam MT1 e inibem MT2 exibiram um efeito antitumoral robusto *in vitro* e *in vivo*, destacando o potencial de tais receptores como alvos terapêuticos.

Palavras-chave: heterogeneidade celular intratumoral, plasticidade epigenética, Eixo Imune-Pineal, melatonina extrapineal, receptores de melatonina, gliomas.

Abstract

Tumor cell plasticity and heterogeneity are key features underlying disease progression and therapeutic resistance. Recent advances of single-cell RNA-seq (scRNA-seq) technologies have highlighted the co-existence of transcriptionally distinct subpopulations of malignant cells within single tumors of different lineages. Thus, there is now a need to establish frameworks to better understand the biological and clinical relevance of such cellular diversity, as well as its underlying molecular mechanisms. To address this issue, we generated scRNA-seq data for 198 cell lines (22 cancer types) and systematically characterized intra-cell line expression heterogeneity. We found that co-existence of highly distinct subpopulations within the same cell line is rare, while continuous patterns of expression heterogeneity, represented by spectra of cellular states are common, recur across different cell lines, are associated with multiple biological process, and are usually independent of genetic diversity. Notably, despite the absence of a native and spatially-variable microenvironment in vitro, many of the continuous programs observed in cell lines recapitulate those found in clinical samples, suggesting a prominent role of intrinsic epigenetic plasticity in generating intratumoral heterogeneity. The data also allowed us to prioritize specific cell lines as model systems of cellular plasticity. As an example, we selected two of such models to demonstrate the temporal dynamics and vulnerabilities associated with a cancer senescence program observed both in cell lines and in human tumors. Additionally, given the means by which malignant cells communicate in the tumor bulk, i.e. physical cell-to-cell interactions and secretion of soluble factors, we also aimed to explore new autocrine/paracrine signaling networks that could be exploited clinically. Melatonin, best known as the “pineal hormone”, is a pleiotropic molecule produced by many extrapineal tissues and increasingly recognized as a tumor suppressor agent. Melatonin acts through several biological mechanisms, including direct scavenging of free radicals and activation of high-affinity G-protein coupled receptors (MT1 and MT2). Over the past decade, our group has provided compelling evidences that fine-tuning the extrapineal production of melatonin during acute inflammation is critical for the maintenance of tissue homeostasis. However, our knowledge about the pathophysiological role of local melatonin in malignant processes is very limited. Interestingly, here we demonstrated that the ability of gliomas to synthesize and accumulate melatonin negatively correlates with their overall malignancy. Using gene expression data, we designed a predictive model of the content of melatonin in the tumor microenvironment, the ASMT:CYP1B1 index, which was shown to be a positive prognostic factor, independent of glioma grade and histological subtype. Finally, as we sought to provide further support for the rational use of melatonin and analogous in brain cancer therapy, we demonstrated that in gliomas and medulloblastomas MT1 and MT2 melatonin receptors play opposite roles in disease progression. Remarkably, compounds that simultaneously activate MT1 and inhibit MT2 displayed a robust antitumor effect in vitro and in vivo, highlighting the potential of such receptors as therapeutic targets.

Keywords: intratumoral cellular heterogeneity, epigenetic plasticity, Immune-Pineal Axis, extrapineal melatonin, melatonin receptors, gliomas.

Introduction

Tumors as complex ecosystems

Cancer is increasingly recognized as a community in which malignant and non-malignant cells interact over time in a dynamic and often tumor-promoting manner (Balkwill et al., 2012). The diversity of these cells is a fundamental feature of tumor ecosystems and ultimately governs disease progression and clinical outcome (Binnewies et al., 2018; Hanahan & Coussens, 2012; Quail & Joyce, 2013). Tumors are infiltrated with different cell types, including fibroblasts, immune and endothelial cells, which collectively form the tumor microenvironment (Balkwill et al., 2012). In addition, malignant cells are themselves heterogeneous, what poses a significant challenge to cancer therapy, as subpopulations of treatment resistant cells may underlie tumor recurrence and metastasis (Marusyk et al., 2012; McGranahan & Swanton, 2015; Pribluda et al., 2015). Various factors contribute to intratumoral heterogeneity, including genetic diversity, as subclonal mutations accumulate during tumor evolution (Gerlinger et al., 2012); spatial variability from extrinsic factors (e.g. oxygen and nutrients; Berglund et al., 2018); and intrinsic epigenetic plasticity related to developmental pathways, like those linked to stem cell differentiation (Tirosh, Venteicher, et al., 2016).

The comprehensive characterization of all cellular elements in the tumor ecosystem has been limited by technical reasons. Although methods such as standard multicolor flow cytometry/immunohistochemistry might allow the identification of distinct subpopulations of cells, they can hardly profile more than 10 proteins simultaneously (Dixon et al., 2015; Pedreira et al., 2013). Conventional DNA and RNA sequencing, on the other hand, provide genome-wide data and have shaped much of our current understanding of human tumors. However, protocols require bulk samples as input material, thereby revealing average cellular patterns (Ren et al., 2018; Tirosh & Suvà, 2019). Recent advances in single-cell sequencing have led to a paradigm shift in cancer research by allowing the study of human tumors at the resolution of individual cells. Over the past decade, single-cell high-throughput technologies have improved considerably from the initial proof-of-principle studies using chips or plates with limited cell numbers (Tang et al., 2009). For instance, recently developed systems based on droplet microfluidics that enable the profile of an

order-of-magnitude-more cells in each batch are gradually becoming standard in the field (Macosko et al., 2015). Despite the dramatic advances, substantial limitations and challenges still exist, including the requirement of fresh tumor samples and highly optimized processing protocols to maximize cell viability and data quality, and the reduced sequencing coverage (i.e. number of genes detected per cell; Ren et al., 2018; Tirosh & Suvà, 2019).

One of the ultimate goals of single-cell analyses is to link tumor subpopulations to distinct cell phenotypes, what is often accomplished using RNA sequencing. For example, studies of adult IDH-mutant oligodendrogliomas/astrocytomas (Tirosh, Venteicher, et al., 2016; Venteicher et al., 2017) and H3K27M-mutant pediatric midline gliomas (Filbin et al., 2018) suggest that such tumors harbor a developmental hierarchy, with stem-like cells capable of self-renewal and more differentiated cells that do not cycle. In melanomas, a subset of cells expressing high levels of the receptor tyrosine kinase AXL, a putative marker of drug resistance, and low levels of the master melanocyte transcriptional regulator MITF and its target genes (TYR, PMEL and MLANA), were shown to be resistant to RAF and MEK inhibitors (Tirosh, Izar, et al., 2016). Notably, the proportion of AXL-high cells appeared to increase in both tumors and cell lines upon treatment and recurrence (Tirosh, Izar, et al., 2016). Finally, in head and neck squamous cell carcinoma (HNSCC), cells with a prominent signal for partial epithelial-mesenchymal transition are located at the periphery of tumors and likely compose the invasive edge (Puram et al., 2017).

Networks of cell-to-cell communication

As multicellular organisms evolved, cells acquired diverse modes of cell-to-cell communication that allow the coordinated functioning of tissues (Pires da Silva & Sommer, 2003). Such interactions are critical for early embryonic development (Eichmann et al., 1997), as well as for the maintenance of adult organs (Chow et al., 2011; Festa et al., 2011). Intercellular communication relies on ligand-receptor interactions, where the ligand can either be a secreted soluble factor, or membrane-bounded, requiring physical proximity between the interacting cells (Pires da Silva & Sommer, 2003). Interactions trigger a chain of specific molecular events inside the cell, which ultimately leads to changes in transcription, translation, as well as modifications in protein folding, processing and localization. The specificity of such pathways depends on the

intensity of the signal and the cross-regulatory interactions with overlapping signal transduction cascades (Dumont et al., 2001).

Signaling by secreted molecules are frequently divided into three general categories according to the distance over which signals are transmitted. In endocrine signaling, the molecules (hormones) are secreted by specialized endocrine cells and carried through the circulation to act on target cells at distant body sites (Lodish et al., 2008). In humans, more than 30 different hormones are produced by endocrine glands, including the thyroid, adrenal, pituitary and pineal (Norman & Litwack, 1997). In autocrine and paracrine signaling, molecules act locally, activating receptors on the same cell and adjacent cells, respectively (Lodish et al., 2008). A recent study revealed that most cell types express tens to hundreds of ligands and receptors, creating a highly connected network of intracellular signal transduction (Ramilowski et al., 2015). More importantly, approximately two thirds of soluble ligands seem to be co-expressed with at least one of its cognate receptors in the same cell, revealing the potential extent of autocrine signaling (Ramilowski et al., 2015). Notably, disrupted autocrine/paracrine cell-to-cell interactions are increasingly recognized as major players controlling tumor progression (Butera et al., 2018; Pietras & Östman, 2010).

The soluble fraction of the tumor microenvironment include signaling molecules such as chemokines, interferons, interleukins, growth and angiogenic factors, which are synthesized by tumor, stromal and/or immune cells (Balkwill et al., 2012). Pro-inflammatory interleukins such as IL6 and IL8, produced either by malignant or stromal cells, have been shown to participate in feed-forward loops involving the nuclear factor κ B (NF κ B) and signal transducer and activator of transcription 3 (STAT3) oncogenic pathways and to promote tumor proliferation, survival, invasion and metastasis (Jayatilaka et al., 2017; Waugh & Wilson, 2008). Moreover, amplified activation of the endothelial growth factor receptor (EGFR), frequently observed in glioblastoma, lung and breast cancer, is a well-known driver of tumorigenesis and results from gene amplification, activating point mutations, and ligand overproduction (Sigismund et al., 2018). There are also factors such as TGF- β that play a complex and paradoxical role, varying by cell type and stage of tumorigenesis (Morikawa et al., 2016). In early stages, TGF- β may act as a tumor suppressor, inhibiting cell cycle progression and promoting apoptosis (Moses et al., 1990). Later, it may enhance invasion and metastasis by inducing epithelial-mesenchymal transition (Roberts & Wakefield, 2003).

Such signaling pathways represent cancer dependencies that can be exploited therapeutically. In this regard, the use of tyrosine kinase inhibitors (TKI) in patients with non-small cell lung cancer has provided the main example of the successes in targeting such oncogenic addiction, while disclosing the challenges of overcoming tumor resistance mechanisms (Murtuza et al., 2019). Tumors with activating EGFR mutations, approximately 15 to 20% of the cases, are sensitive to EGFR tyrosine kinase inhibitors (Pao & Chmielecki, 2010); and a paradigm shift emerged when these compounds showed improved efficacy to chemotherapy as initial therapy (Zhou et al., 2011). However, despite revolutionary advancements, patients who were sensitive to first- and second-line EGFR TKIs eventually progressed after approximately 9 to 11 months, due to acquisition of new EGFR mutations such as T790M (Sequist et al., 2011). Third-generation TKI osimertinib which precisely targets EGFR T790M resistant clones was recently granted full FDA approval, with data that demonstrate significant improvement in clinical endpoints (Soria et al., 2018). Such compounds illustrate the current focus of much anticancer drug development: targeted therapies interfering with specific molecular pathways that are central to particular cancers. Targeted therapies are a cornerstone of precision medicine and aim to provide more biologically-grounded and patient-tailored treatment regimens (Collins & Varmus, 2015).

Extrapineal melatonin and the Immune-Pineal Axis

As analytical methods increased in sensitivity and specificity, it has become apparent that some of the well-known “endocrine hormones” are not exclusively produced by specialized glands and can act as autocrine/paracrine signals in other tissues. For instance, pioneering works have shown that the local production of adrenal glucocorticoids by thymic epithelial cells play an important role in antigen-specific thymocyte development (Vacchio et al., 1994). Evidences also indicate the existence of extrapancreatic sources of insulin (Kojima et al., 2004) and glucagon (Lund & Knop, 2019). Additionally, during the past two decades, considerable attention has been paid to melatonin, the “pineal hormone”. Melatonin is an ancient amphiphilic molecule present in a multitude of taxa, from bacteria to plants and animals (Tan et al., 2009). It was first isolated in the bovine pineal tissue in 1958 by Aaron B. Lerner, and named after its ability to lighten the skin of frogs by reversing the effects of melanocyte-stimulating hormone (Lerner et al., 1958). For a

number of years after its discovery, melatonin was considered to be exclusively produced by the pineal gland. Almost two decades later, the presence of melatonin synthesis enzymes was described in the retina and cerebellum of pinealectomized rodents (Bubenik et al., 1974; Cardinali & Rosner, 1971). Since then, diverse non-pineal sources of melatonin have been revealed (Acuña-Castroviejo et al., 2014).

Melatonin produced by the pineal gland during the night is best known for its role as the “hormone of darkness”, translating the environmental dark phase to the organism and ensuring the synchronization of circadian and seasonal rhythms (Reiter, 1993). In mammals, melatonin biosynthesis includes four enzymatic steps, beginning with the hydroxylation of tryptophan to 5-hydroxytryptophan, which is then decarboxylated forming serotonin (Zhao et al., 2019). Serotonin is N-acetylated by serotonin N-acetyltransferase (AANAT) to form N-acetylserotonin (NAS). NAS is then converted to melatonin by the enzyme acetylserotonin-O-methyltransferase (ASMT). In the pineal gland, the temporal gating of nocturnal melatonin synthesis is determined by sympathetic innervations controlled by the central biological clock, the hypothalamic suprachiasmatic nuclei (Simonneaux & Ribelayga, 2003). In humans, as well as in rodents, the release of the norepinephrine during the night promotes the phosphorylation of AANAT through a cAMP-dependent, protein kinase A-mediated phosphorylation mechanism (Ackermann & Stehle, 2006). 14-3-3 chaperone proteins bind to PAANAT protecting it against proteasomal degradation, and inducing allosteric changes that result in increased enzymatic activity, and thus enhanced melatonin production (Klein et al., 2002).

Melatonin presumably emerged in primitive unicellular organism as a potent electron donor, detoxifying free radicals generated during the processes of photosynthesis and energetic metabolism (Tan et al., 2009). However, during evolution this indolamine became a pleiotropic molecule, despite its unchanged chemical structure. The broadening of its functional repertoire was accompanied by the emergence of specific binding sites/receptors and associated signaling transduction pathways. In mammals, many of the currently known activities of melatonin are mediated by the high-affinity G-protein coupled receptors MT1 and MT2 located in the plasma membrane (Reppart et al., 1996), although they have recently been detected also in the mitochondrial membrane (Ahluwalia et al., 2018; Suofu et al., 2017). Both receptors are typically coupled to $G_{i/o}$ proteins, inhibiting cAMP/protein kinase A signaling, while MT1 receptors might also be coupled to G_q proteins, activating the phospholipase C pathway (Brydon et al., 1999;

Jockers et al., 2016). In the cytosol, besides reducing oxidative stress, melatonin can also bind to calmodulin (Benítez-King & Antón-Tay, 1993), and to the enzyme quinone reductase 2, often designated as receptor MT3 (Nosjean et al., 2000).

Currently, known extra-pineal sources of melatonin include the brain, retina, lens, cochlea, Harderian gland, airway epithelium, skin, gastrointestinal tract, liver, kidney, thyroid, pancreas, thymus, spleen, carotid body, reproductive tract, immune and endothelial cells (Acuña-Castroviejo et al., 2014). Extra-pineal melatonin is not released into the circulation, acting locally in the maintenance of tissue homeostasis (Venegas et al., 2012). For instance, over the past decade, our group has provided compelling evidences that during acute inflammatory responses there is a transient switch in melatonin production from the pineal gland to peripheral organs/immunocompetent cells (Markus et al., 2013, 2018, 2007; Markus & Ferreira, 2011). This bidirectional communication, named the Immune-Pineal Axis, provides a framework for understanding the role of melatonin in the mounting and resolution of immune responses (de Oliveira Tatsch-Dias et al., 2013; Pontes et al., 2006).

The nocturnal rise of melatonin is known to impair leukocyte transendothelial migration to the site of tissue damage/infection, a fundamental step of innate immunity (Lotufo et al., 2001; Marçola et al., 2013; Tamura et al., 2010). In pinealocytes, pathogen- and danger-associated molecular patterns bind to pattern recognition receptors and promote the nuclear translocation of the transcription factor NF κ B, a classical regulator of inflammation (Carvalho-Sousa et al., 2011; Da Silveira Cruz-Machado et al., 2010). In the nucleus, transactivation domain-free NF κ B dimers p50/p50 block the expression of *Aanat* and hence the nighttime production of melatonin, facilitating leukocyte rolling and adhesion to the endothelial layer at the site of inflammation (Carvalho-Sousa et al., 2011; Da Silveira Cruz-Machado et al., 2010; Fernandes et al., 2006). During inflammation initiation, simultaneous activation of glucocorticoid receptors (GR) and adrenoceptors α_1 and β_1 in the pineal gland contributes to the blockage of melatonin production (Fernandes et al., 2017). During the resolution phase, on the other hand, as the sympathetic tonus decreases, GR activation (Fernandes et al., 2017), associated with others immune signals such as interferon-gamma (Barbosa Lima et al., 2019), induces *Aanat* transcription and allows the restoration of the nighttime production of melatonin.

At the site of inflammation, activated macrophages start to produce melatonin upon induction of *Aanat* expression by NF κ B dimers containing transactivation domains (p50/RelA and cRel/RelA) (Muxel et al., 2016, 2012; Pires-Lapa et al., 2013). In this context, melatonin acts autocrinally/paracrinally reducing oxidative stress, potentiating phagocytoses and inhibiting bacterial proliferation. Similarly, acute neuroinflammation induced by lipopolysaccharide injected directly into the lateral ventricles of adult rats promotes the synthesis of melatonin by astrocytes and microglia in the cerebellum, though not in the cortex or hippocampus (Pinato et al., 2015). Interestingly, lipopolysaccharide treatment leads to neuronal death in the hippocampus and cortex, but not in the cerebellum. This privileged protection of cerebellar cells is abrogated by blocking melatonin receptors, suggesting that the local production of melatonin protects cerebellar neurons from neuroinflammation-induced cytotoxicity (Pinato et al., 2015).

Melatonin and cancer

In 2007, based on robust mechanistic evidence coming from well-designed experimental studies, the International Agency for Research on Cancer Working Group classified night shift work involving circadian disruption as probably carcinogenic to humans (Group 2A) (IARC Monographs Vol 124, 2019). For instance, exposure to constant light was shown to induce the development of metabolic syndrome and spontaneous tumorigenesis, and shorten the life span of both male and female rats, adverse effects shown to be prevented by the administration of melatonin in nocturnal drinking water (Anisimov et al., 2012). In human breast cancer xenografts, exposure to increasing intensities of white fluorescent light during each 12-hour dark phase result in a dose-dependent suppression of nocturnal melatonin blood levels and a stimulation of tumor growth and linoleic acid uptake/metabolism to the mitogenic molecule 13-hydroxyoctadecadienoic acid (Blask et al., 2005). Importantly, human breast cancer xenografts perfused in situ with physiologically melatonin-rich blood collected from health female volunteers during the night exhibited markedly suppressed proliferative activity compared to those perfused with melatonin-depleted blood collected following exposure to light at night (Blask et al., 2005).

Over the past 30 years, accumulating evidence has outlined the relevance of melatonin to human physiology and pathology. Slow release melatonin (Circadin) and several melatonin

analogues (e.g. Ramelteon, Agomelatine and Tasimelteon) are currently used clinically for the resynchronization of circadian rhythms during jet lag and shift work, and for managing insomnia, depression, and non-24-h sleep-wake disorder (Liu et al., 2016). Additionally, the potential of melatonin as an anticancer agent has gained increasing attention, and the growing number of supporting in vitro and in vivo experimental studies include a wide variety of tumors, such as, melanoma, glioma, sarcoma, as well as cancers of breast, ovary, cervix, and prostate (Cutando et al., 2012; Li et al., 2017). As a pleiotropic molecule, melatonin has been documented to act through several biological mechanisms, including: inhibition of glioma cell migration/invasion by direct scavenging intracellular free radicals and impairing the reactive oxygen species-dependent NF κ B oncogenic signaling (Wang et al., 2012); induction of apoptosis of colorectal cancer cells through inactivation of calmodulin-dependent protein kinase II, followed by dephosphorylation and nuclear import of histone deacetylase 4 (Wei et al., 2015); and suppression of estrogen receptor alpha mRNA expression and transcriptional activity in breast cancer cells via MT₁ receptor activation (Girgert et al., 2009; Mao et al., 2010).

Despite the extensive list of promising basic research studies conducted in the past decades, at present, completed clinical trials focusing on melatonin and cancer sum up to only 20, 16 of which were carried out at the Institute of Biological Medicine in Milan, Italy (González et al., 2019). Overall, melatonin seems to effectively improve the quality of life of patients receiving radio and/or chemotherapy by normalizing sleep and relieving symptoms such as pain, asthenia, and anorexia (González et al., 2019). Although individual trials with hepatocellular carcinoma (Yan et al., 2002), glioblastomas (Lissoni et al., 1996), colorectal (Cerea et al., 2003), and lung cancers (Lissoni et al., 2003) have suggested that melatonin, given as an adjuvant, can also significantly increase tumor remission rate and patient overall survival, data are still limited and show inconsistencies in more recent studies (Berk et al., 2007; Sookprasert et al., 2014). There is thus a clear necessity for further large-scale and multi-center randomized clinical trials. Additionally, as rational therapies that interfere with specific molecules become the focus of anticancer drug development, the roles of MT₁ and MT₂ melatonin receptors as relevant targets should be explored.

Objectives

Over the past few years, single-cell RNA-seq (scRNA-seq) studies of different cancer types have highlighted the heterogeneity among malignant cells within single tumors. Thus it is now crucial to establish frameworks to better understand the biological and clinical relevance of such cellular diversity, as well as its underlying molecular mechanisms. In this sense, although human cell lines have been a cornerstones of cancer research, their value as models of intratumoral heterogeneity remains largely unknown. To comprehensively address this issue, we generated scRNA-seq data for 198 cell lines (22 cancer types, 53,513 cells) from the Cancer Cell Line Encyclopedia (CCLE) collection and systematically characterized intra-cell line expression heterogeneity. We uncovered recurrent programs of heterogeneity and i) determined their similarity to programs of intratumoral heterogeneity previously described in clinical samples; ii) evaluated their association with mutations and drug sensitivities; and iii) investigated their dependency on genetic variability. Finally, we select two optimal cell lines to model a recurring program associated with partial epithelial senescence. We demonstrated the dynamics, regulation, and vulnerabilities associated with this program, highlighting its role in driving phenomena such as drug resistance.

Additionally, we aimed to explore autocrine and paracrine signaling mediating the communication among the diverse malignant cells in the tumor ecosystem, thereby looking for new molecular networks that could be exploited clinically. It is now clear that fine-tuning the extrapineal production of melatonin is critical for the maintenance of an internal steady state within tissues, as illustrated by the Immune-Pineal Axis. However, our knowledge of the pathophysiological role of local melatonin in malignant processes remains very limited. Thus, here, we comprehensively characterized the melatonergic system of human gliomas, the most common primary brain tumor in adults and one of the deadliest malignant neoplasms. We investigated the association between the capacity of glioma cells to synthesize and accumulate melatonin in the surrounding microenvironment and their overall malignancy as well as patient survival. We also further elucidated differential effects of MT1 and MT2 receptors activation, highlighting the therapeutic potential of specific melatonergic agonists/antagonist in the treatment of brain cancers.

Conclusions

Causes and consequences of tumor cell plasticity and heterogeneity

Tumor cell plasticity and heterogeneity are key mechanism underlying disease progression and therapeutic resistance (Dagogo-Jack & Shaw, 2018). Recent advances of single-cell technologies have allowed the profiling of tumors at unprecedented depth, enabling the dissection of potential cell trajectories associated with changes in phenotypes (Neftel et al., 2019; Peng et al., 2019; Puram et al., 2017; Tirosh, Izar, et al., 2016; Tirosh, Venteicher, et al., 2016; Venteicher et al., 2017; Zhang et al., 2019). Nevertheless, the molecular mechanisms underlying such changes and ways of modulating it remain poorly understood. Most studies of intratumoral heterogeneity have focused on genetic alterations, as genome instability represents a prominent fuel for cell-to-cell variation and hence selection and evolution (Campbell et al., 2010; de Bruin et al., 2014; Gerlinger et al., 2012; Harbst et al., 2016; Jamal-Hanjani et al., 2017; Yates et al., 2015). However, as evidenced by our work, even genetically homogenous cells show significant expression heterogeneity. Remarkably, by analyzing scRNA-seq data of dozens of cell lines from diverse cancer types we demonstrated that most heterogeneity observed within tumors reflect non-genetic mechanisms that generate continuous programs of expression diversity, characterized by spectra of cellular states.

Expression heterogeneity of genetic homogeneous cellular populations, could reflect, for instance: i) the intrinsic fluctuations due to the randomness inherent to transcription; ii) epigenetic developmental programs; and iii) responses to microenvironmental factors. Interestingly, we showed that many of the continuous programs observed in cell lines are recurrent and recapitulate those seen in fresh human tumors, even though in vitro conditions lack a native and spatially-variable microenvironment. These observations suggest that some cellular developmental hierarchies present in normal tissues, such as those observed during wound-healing and cellular aging, are at least partially preserved in tumors (and cell lines), representing a prominent source of phenotype variability. As illustrated by experiments with the HNSCC cell lines JHU006 and SCC47, which harbor a variability program of epithelial senescence (EpiSen) also seen in clinical

samples of HNSCC, such non-genetic heterogeneity is reversible and dynamic, and could thus more directly regulate cellular phenotypes.

Non-genetic variability can also contribute to therapeutic resistance by multiple different mechanisms. For instance, we demonstrated that cells with high expression of the EpiSen program are more sensitive to inhibitors of the PI3K/AKT pathway and less sensitive to inhibitors of the cell cycle when compared to the EpiSen low population. Thus, epigenetic manipulation of cancer cells that makes expression patterns more uniform could be a powerful therapeutic tool to improve the efficacy of targeted/chemo therapies by reducing the risk of resistant phenotypes. Notably, by showing the relevance of certain cell lines as models of intratumoral heterogeneity, we provided the framework for the scientific community to start testing such innovative hypothesis. In coming years, major challenges will likely involve the transferring of the acquired knowledge "from bench to bedside". More specifically, developing cost- and time-effective ways to assess expression heterogeneity in clinical samples, as well as generating protocols on how to use this information to guide therapeutic decisions.

The melatonergic system in cancer prognosis and treatment

Over the past decades, melatonin, best known as the “hormone of darkness” or the “pineal hormone”, has been revealed as a pleiotropic molecule with diverse biological functions: it is produced by multiple tissues (Acuña-Castroviejo et al., 2014); has clinical relevance as a treatment for conditions such as insomnia and Alzheimer’s disease (Liu et al., 2016); and even shows anticancer properties (Cutando et al., 2012). Adding to this list, here, we are the first to show the role of glioma-synthesized melatonin as an autocrine/paracrine suppressor of disease progression. Remarkably, analysis of RNA-seq data of more than 4,500 samples from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx), revealed that in almost all cancer types the content of melatonin locally produced and accumulated, as predicted by the ASMT:CYP1B1 expression index, is significantly lower than in matched normal tissues (**Fig. 1**). Accordingly, as observed in gliomas, a low index value, indicative of decreased melatonin, is associated with poor prognosis in bladder urothelial carcinoma, colorectal adenocarcinoma, medulloblastoma, metastatic melanoma, lung squamous cell carcinoma, pancreatic adenocarcinoma, pheochromocytoma/paraganglioma, and stomach adenocarcinoma (**Fig. 2**).

Figure 1. Melatonin levels, as predicted by the ASMT:CYP1B1 expression index, in normal and malignant tissues. The index value was calculated for each sample according to the mRNA levels of melatonin synthesis (ASMT) and metabolism (CYP1B1) enzymes, as previously described (Kinker et al., 2016). Comparisons were performed using the two-sided Students t-test. Gene expression data were downloaded from public databases (GTEx and TCGA).

Figure 2. The prognostic value of the ASMT:CYP1B1 index in solid tumors. Kaplan-Meier survival curves of patients with tumors presenting low vs. high ASMT:CYP1B1 index scores. The index value was calculated for each sample based on the mRNA levels of the melatonin synthesis (ASMT) and metabolism (CYP1B1) enzymes, as previously described (Kinker et al., 2016). Gene expression and clinical data were obtained from public database repositories (Cho et al., 2011, for medulloblastomas, and TCGA, for all other tumors).

Although further studies are needed to define if decreased levels of tissue melatonin are a cause or consequence of tumorigenesis, we can speculate about the role of this indolamine as a major modulator of the malignant behavior. Given the tissue- and context- specific effect of melatonin receptors activation, it is possible that this apparent universal trend is linked to the more primitive role of melatonin as a broad-spectrum antioxidant (Tan et al., 2009). Reactive oxygen species (ROS) can be produced from endogenous sources, including organelles such as mitochondria, peroxisomes and endoplasmic reticulum, where the oxygen consumption is high, as well as from pollution, alcohol, tobacco smoke, and UV radiation (Di Meo et al., 2016). Keeping the cellular redox state well-balanced is essential for the maintenance of tissue homeostasis, and it is now clear that ROS have a dual role in cancer (Reczek & Chandel, 2017). Slightly increased levels of ROS can promote genomic instability and activate pro-tumorigenic signaling such as NF κ B (A. C. H. Chen et al., 2011). Too high concentrations, on the other hand, can cause excessive oxidative damage and cell death (Y. Chen et al., 2008). Thus, locally produced melatonin might

act, together with others known antioxidant systems (e.g. thioredoxin and glutathione systems), as a buffer, ensuring optimal levels of free radical in the tissues. Of note, recent studies with neuronal cells (Suofu et al., 2017) and oocytes (He et al., 2016) have shown that melatonin can be synthesized in the mitochondria, where it plays a role avoiding electron leakage.

As described earlier, apart from functioning as an electron donor, melatonin can also activate high-affinity G-protein coupled receptors, MT1 and MT2. Although this indolamine is increasingly referred as a potential adjuvant in cancer treatments, few serious attempts have been made to understand the effects associated with the activation of each one of these receptors. Most in vitro studies use concentrations of melatonin that are much higher than those required for the saturation of MT1 and MT2 receptor binding sites, and thus likely trigger many receptor-independent effects. Importantly, in patients, achieving high concentrations of melatonin in the tumor microenvironment through systemic administration might be a challenge, limiting the reproduction of such in vitro observations in the clinics. Moreover, as anticancer drug discovery moves towards the development of more rational and biologically-grounded treatments, it becomes clear that the comprehensive characterization of targeted molecular networks is essential for improving therapeutic efficacy while avoiding adverse effects. In this regard, surprisingly, we showed that in gliomas and medulloblastomas MT1 and MT2 play opposite roles in controlling disease progression. Compounds that activate MT1 and inhibit MT2 displayed a remarkable growth suppressive effect in glioma xenografts, while the therapeutic benefits of melatonin were variable and with no significant trend. All together, we provided the first robust evidence of the therapeutic relevance of MT1 and MT2 receptors as druggable targets in brain cancer, which now must be tested clinically.

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