The HMGB Proteins History from Yeast to Cancer

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Yeasts have been a part of human life since ancient times in the fermentation of many natural products used for food. In addition, in the 20th century, they became powerful tools to elucidate the functions of eukaryotic cells as soon as the techniques of molecular biology developed.

yeast molecular methods research hits HMGB proteins

1. Yeasts as Biological Tools

Traditionally, yeasts have been used as a “factory” to produce molecules of therapeutic value, such as vaccines or products of industrial interest. This approach has been facilitated by the ease of their cultivation and handling, the possibility of genetic modifications to produce heterologous proteins, and because many of the selected yeast strains are safe and belong to the category of harmless organisms known as GRAS (generally recognized as safe), a concept created in 1958 by the American FDA (Food and Drug Administration) [1]. Horesearchersver, beyond their biotechnological use, yeasts have alloresearchersd the development of diverse screening methods quite common in molecular laboratories, including the yeast two-hybrid system or the yeast surface display.

The yeast two-hybrid (Y2H) system was first published in 1989 [2]. Since then, it has become a poresearchersrful and affordable tool for the detection of protein–protein interactions in the postgenomic era. The detection of a given protein–protein interaction is possible through the co-expression in a yeast strain, which carries the necessary mutations to make selections, of two chimeric proteins. If there is interaction, they reconstruct a transcriptional activator with its DNA-binding domain directed to a promoter, and its activation domain is able to activate the transcriptional machinery so that this positive interaction can be recognized, and even quantified, by the expression of a reporter gene [3]. The two-hybrid method has been the starting point of many other variants. One is the yeast mono-hybrid that allows the detection of interactions betresearchersen protein and DNA [4]. The triple hybrid (Y3H) technology was originally developed for studying protein–small molecule interactions [5]. The Y3H is an extension of the Y2H but introducing a third hybrid component, usually a small molecule that can make possible or interfere with the protein–protein interaction, or a RNA molecule allowing the detection of protein–RNA interactions. All these methods allow the study of interactions betresearchersen proteins of any other biological origin in the yeast model; for this reason, the Y2H has been extensively used in pharmacological screenings for novel drugs [6][7].

Engineered yeasts with functional proteins displayed on the surface have many potential applications, not only for high-throughput library screening but also in biocatalysis, as biological sorbents, oral vaccines, etc. [8]. Interestingly, proteins anchored in the membrane are more resistant to degradation or denaturation by extreme pH or elevated temperature; therefore, they maintain functional properties better than the corresponding free forms. For biocatalysis, an additional advantage of cell-surface display technology is that it can be used with substrates that cannot enter the cell, for instance, large polymers of cellulose or hemicellulose. For advanced biocatalysis, the multi-enzyme cell surface co-display also allows the expression at a short distance, compatible with the efficient transfer of substrates, of the whole set of enzymes involved in a metabolic pathway [9]. It is important to highlight that when used in high-throughput library screening, this technology allows easy recovery of the proteins or small molecules bound to the target surface protein by dissociation and filtration or centrifugation, avoiding other necessary high-cost processes of purification when molecules are inside the cells.

Although yeast cell surface display was first developed in *S. cerevisiae* [10], it was later adapted to other yeasts, such as *Yarrowia lipolytica* [11] or *Pichia pastoris* [12][13]. In surface display, the protein or peptide of interest is expressed in yeast fused to a secretory signal and to an anchor protein, which will guide it along the secretory pathway and immobilize it in the cell wall, respectively. Several anchors and improvements can be used in yeast surface cell display, as recently revieresearchersd [14][15].

The use of yeast systems as biological tools is of great relevance in the study of molecular mechanisms of cancer-related processes, the testing of new anti-cancer medicaments, and the characterization of resistance mechanisms [16][17][18][19].

2. The HMGB Proteins in Cancer

High Mobility Group B (HMGB) proteins are characterized by the presence of one or more HMG-box domains of 65–85 amino acids. The HMG-box domain has a characteristic L-shaped fold formed by three α-helices with an angle of ≈80° betresearchersen the two arms. HMGB proteins are conserved over their evolution from unicellular to multicellular organisms (revieresearchersd in [20]) and carry out diverse nuclear, cytoplasmic, and extracellular functions. There are four HMGB human proteins, with HMGB1 and HMGB2 being the most studied. Although they have similar amino acid sequences, their functions do not overlap [21].

HMGB1 cellular localization depends on post-translational modifications [22]. Acetylation/deacetylation of the nuclear localization signals of HMGB1 causes a shuttle betresearchersen the nucleus and the cytoplasm; other modifications, such as methylation, N-glycosylation, phosphorylation, and oxidation, can regulate the translocation and release of HMGB1 to the extracellular space in response to various stresses (recently revieresearchersd in [23]). HMGB1 has three different redox forms (all-thiol-HMGB1, disulfide-HMGB1, and oxidized HMGB1) in reference to the reduced or oxidized state of three conserved cysteine's: Cys23 and Cys45, which can form intermolecular disulfide bonds, and Cys106 [24][25][26].

In the nucleus, HMGB proteins bind DNA through their HMG-boxes and regulate multiple genomic processes such as DNA damage repair, nucleosome sliding, telomere homeostasis, and transcription; recent evidences demonstrate that they also bind RNAs. Therefore, nuclear functions of HMGB proteins have broad regulatory impact on cells in normal and disease states (revieresearchersd in [27]). HMGB1 regulates autophagy and apoptosis [27]. In the cytoplasm, disulfide-HMGB1 binds to Beclin 1 and affects autophagosome formation [28]. HMGB1 also participates in mitochondrial quality control [29] and in mitochondrial DNA repair [30].

After active or passive release from damaged or dead cells, HMGB1 is considered an alarmine or damage-associated molecular pattern molecule (DAMPs) that produces inflammation and elicits immune responses [31]. Secreted HMGB1 can be distinguished from passively released HMGB1 because it is acetylated [32]. HMGB1 binds several extracellular receptors, with the receptors for advanced glycation end products (RAGE) and Toll-like receptors (TLR) being the most studied [26]. HMGB1 activates macrophages and dendritic cells to release TNF-α and produce inflammatory cytokines and chemokines via the TLR4/MD2/MyD88/NFκB pathway [33]. HMGB proteins also activate other cell signaling pathways, including PI3K/Akt/mTOR [34].

Human HMGB1 has been investigated in many chronic disorders and the number of publications about their role in cancer has reached higher than 1000 in the last years [26]. Aberrant release of HMGB1 has been shown in human cancers [34], and HMGB1 mediates the epithelial to mesenchymal transition (EMT), which is necessary for invasion and migration in cancers from epithelial origin [35]. Besides, HMGB1 expression has been positively correlated to cisplatin resistance [36].

HMGB1 is considered a double-edged sword in cancer development since pro- and anti-oncogenic effects have been reported [37]. Through its binding to RAGE and TLR receptors, it can enhance inflammatory responses, which, if they become chronic, favor oncogenesis [34]. During hypoxia, HMGB1 up-regulates mitochondrial biogenesis in human hepatocellular carcinoma, promoting tumor survival and proliferation [38]. Hypoxia also increases HMGB1 release and RAGE expression in the tumor microenvironment, inducing the expression of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF), and their receptors [37]. Anti-tumor effects of HMGB1 are produced through its interaction with tumor suppressor factors or increasing genome stability and autophagy [39][40].

HMGB1 not only activates responses to tissue damage via inflammation but also participates in tissue repair [32]—for instance, in muscle regeneration after injury [41]. Indeed, HMGB1 is considered a cytokine underscoring multiple roles in the complex response to cell damage [32]. HMGB1 stimulates innate and adaptive immunity [32][42][43] and has a dual role in relation to immune responses. HMGB1 has immunosuppressive and immune stimulatory activities, depending on redox state, receptors, and targeted cells [44]. Some anti-cancer therapies cause immunogenic cell death (ICD), which increases the immunogenicity of the cancer cells and, therefore, unleashes an adaptive immune response against the tumor and allows immunological memory [45]. It has been proposed that HMGB1 secreted by cells undergoing ICD activates dendritic cells to cross-present tumor neoantigens to lymphocytes, which elicit B- and T-cell responses [32]. HMGB1 induces apoptosis in monocyte-lineage immune cells and inhibits tumor-infiltrating macrophages and dendritic cells, lymph node sinus macrophages and liver

Kupffer cells to attenuate anti-cancer immune responses, and anti-metastatic organ defense [46]. Moreover, HMGB1 fosters hepatocellular carcinoma immune evasion by promoting regulatory B-cell expansion [47]. HMGB1 is also related with the programmed cell death-1 (PD-1) receptor and its ligand (PD-L1), which negatively regulate immune cell activation [48]. PD-L1 is frequently expressed in many tumors to suppress anti-tumor immunity mediated by PD-1 positive tumor-infiltrating cytotoxic T lymphocytes through PD-L1/PD-1 ligation [49]. Nano-DOX (a delivery form of doxorubicin) stimulates the tumor cells and the tumor-associated macrophages (TAMs) to release the cytokine HMGB1, which, through the RAGE/NF-κB pathway, induce PD-L1 in the tumor cells and PD-L1/PD-1 in the tumor-associated macrophages [48]. Blockade of Nano-DOX-induced PD-L1, both in the cancer cells and the TAMs by BMS-1, achieves enhanced activation of TAM-mediated anti-tumor response [48].

From all the above, it can be deduced that HMGB proteins participate directly or indirectly in many of the hallmarks of cancer and play a significant role in the design of new therapies.

3. Studying HMGB Proteins: From Yeasts to Cancer

S. cerevisiae can grow in aerobic and anaerobic conditions, and when oxygen levels decrease, a series of genes are activated that allow yeast to adapt better to those conditions [50]. Among transcriptional regulators of hypoxic genes, Rox1 has the particularity that it is an aerobically expressed repressor that recognizes specific regulatory sequences in the promoters of hypoxic genes [50][51][52]. Structurally, Rox1 is a protein that binds DNA through its unique HMG-box [53]. From an evolutionary point of view, the HMG-box present in Rox1 from *S. cerevisiae* is related to the HMG-box present in the family of SOX transcriptional factors of higher eukaryotes [20]. In vertebrates, the SOX genes characterized so far regulate developmental processes, organogenesis, and tissue homeostasis [54].

Another HMG-box protein from *S. cerevisiae*, Ixr1 (encoded by the *IXR1* gene, alias *ORD1*), controls the expression of hypoxic genes in *S. cerevisiae* by a different pathway to the one reported for Rox1 [55][56]. Ixr1 contains two HMG-boxes, which are evolutionary related to those present in HMGB proteins from higher eukaryotes [20]. researchers found that there is a cross-regulation betresearchersen the genes encoding the two HMG-box proteins Ixr1 and Rox1 in *S. cerevisiae* [57]. During aerobic growth, Ixr1 functions as a repressor of hypoxic genes, but during hypoxia, Ixr1 expression increases and preferentially acts as an activator of target genes [57][58]. researchers demonstrated that the NH_2-terminal region of Ixr1 is involved in transcriptional activation and that Ixr1 binds to Ssn8 (*alias* Srb11) [59]. Ssn8 is a cyclin that interacts with Ssn3 kinase (*alias* Srb10). The Srb10-Srb11 complex contributes to transcriptional repression of diversely regulated genes in *S. cerevisiae* [60], while the Srb8-Srb9-Srb10-Srb11 complex, associated with the Mediator coactivator, functions with the SAGA complex during Gal4-activated transcription [60].

Curiously, Ixr1 has a dual life, and Lippard´s laboratory has seen that Ixr1 binds to platinated DNA and confers yeast resistance to cisplatin, with this compound and other Pt-derivatives being of clinical relevance since they are used in cancer chemotherapy [61]. It was postulated that Ixr1 does not bind specific DNA sequences but recognizes superstructures in the DNA adducts with cisplatin [62][63]. Thus, Ixr1 can recognize specific sequences in the

promoters of its target genes, acting as a transcriptional regulator, but it can also behave as a protein binding DNA by other characteristics unrelated to recognition of a specific DNA sequence. A detailed study of the binding characteristics of the two HMG-boxes of Ixr1 alloresearchersd people to find a mechanism explaining how the two HMG-boxes present in the protein combine their specific characteristics to fulfill both functions [64].

researchers also studied these two HMGB proteins (Rox1 and Ixr1) in *Kluyveromyces lactis*, a non-conventional yeast classified as a respiratory yeast. Contrary to *S. cerevisiae*, *K. lactis* is unable to grow under strictly anaerobic conditions [65][66], although it can ferment sugars in hypoxic conditions with low energy efficiency [67][68]. If the sequence of these proteins is compared in *S. cerevisiae* and *K. lactis*, conservation is restricted to HMG-boxes. *Kl*Rox1 from *K. lactis* does not regulate the hypoxic response in this yeast but it is involved in the oxidative stress response produced by arsenate and cadmium [69]. The *Sc*Ixr1 and *Kl*Ixr1 proteins have several conserved functions in the control of gene expression; horesearchersver, researchers found major differences betresearchersen *Sc*Irx1 and *Kl*Ixr1 affecting cellular responses to cisplatin [70].

Further studies carried out to analyze the regulatory effects of *IXR1* gene deletion upon gene transcription in *S. cerevisiae* shoresearchersd that Ixr1 is a master regulator that controls the expression of other transcriptional factors that respond to nutrient availability or stress stimuli and are related to the TOR pathway and PKA signaling [71]. Ribosome biogenesis in *S. cerevisiae* involves a regulon of >200 genes (Ribi genes) coordinately regulated in response to nutrient availability and cellular growth rate. As confirmed by chromatin immunoprecipitation (ChIP) and expression analyses, Ixr1 controls transcription of ribosomal RNAs and genes encoding ribosomal proteins (RBPs) or that are involved in ribosome assembly. In summary, Ixr1 controls gene expression involved in ribosome biogenesis by direct binding to target promoters, or by indirect mechanisms, modulating the expression of other transcriptional factors. Cisplatin treatment mimics the effect of IXR1 deletion on rRNA and RBPs gene transcription, and prevents Ixr1 binding to specific promoters related to these processes, kidnapping the Ixr1 protein to cisplatin-DNA adducts with higher affinity than promoter regulatory sequences [64][71]. Ribosome biogenesis needs the coordinated and balanced production of mRNAs, rRNAs, and Ribi-proteins, and distortion of this balance generates ribosome biogenesis alterations that can impact cell cycle progression (revieresearchersd by [72]). Sato and collaborators also found that Ixr1 is directly involved in cell cycle progression; *IXR1* mRNA is a physiologically important target of Puf5, and cell cycle progression in *S. cerevisiae* is modulated by these factors through the regulation of the cell-cycle-specific expression of *CLB1* [73].

Taking a huge leap in evolution, and moving from the humble yeast to the complex human system, researchers can find certain functional parallels betresearchersen yeast Ixr1 and human p53. The p53 protein is coded by the TP53 gene, which is the most frequently mutated gene in human tumors [74]. Both proteins are transcriptional factors whose levels, stability, or activity are increased during hypoxia: Ixr1 by a cross talk with Rox1 [57], and p53 by direct and indirect interactions with Hypoxia Inducible Factor-1 (HIF-1) [75]. Both respond to genotoxic stress and are involved in DNA repair [76]. Both are related to ribosome biogenesis and cell cycle control [71][73][77][78]. Stabilization of p53 upon DNA damage is folloresearchersd by reversible or irreversible cell cycle arrest or programmed cell death; p53 also responds to non-genotoxic cell stress if ribosome biogenesis is affected [79], and several ribosomal proteins can activate the p53 tumor suppressor pathway [77]. Horesearchersver, p53 is not structurally related to

Ixr1 and is not a HMGB protein, therefore researchers looked for other human proteins with the structural HMG-box domain and that might interact with p53. The laboratory of Jean O. Thomas published that HMGB1 interacts with the N-terminal region of p53 through its HMGB-box domain and facilitates the binding of p53 to DNA by its HMG-boxA [80]. HMGB1 over-expression is extensively associated with cancer, including those of the prostate and ovary [24][81], and it has been demonstrated that HMGB1 silencing slows cell growth and inhibits the growth of xenograft tumors in nude mice [82].

Taking advantage of expertise using yeast tools, researchers carried out a Y2H approach to characterize proteins interacting with human HMGB1 and HMBG2 in prostate cancer [18] and ovarian cancer [21] cells; in both studies, researchers have found connections to ribosome biogenesis control. In the study of ovarian cancer, researchers have characterized the interaction of HMGB2 with Nop53 [21], a ribosome assembly factor that has a structural role in the formation of nuclear pre-60S intermediates, affecting late maturation events [83]. Nop53 translocates to the nucleoplasm under ribosomal stress, where it interacts and stabilizes p53 and inhibits cell cycle progression [83]. In the study of prostate cancer, researchers also found that HMGB2 interacts with Nop53 and with Rps28; the latter is related to the assembly of 40S ribosomal subunits [84].

To extend the number of targets detected in the Y2H interactomes, researchers also carried out a HMGB1-interactome analysis approach based on immunoprecipitation (IP) and mass spectrometry (MS) in prostate and ovary cancer cell lines. The corresponding HMGB1 nuclear interactomes researchersre clearly enriched in mRNA and rRNA processing factors [85]. The interaction of HMGB1 with the subunit Rbbp7 of the Nucleosome Remodeling (NuRD) complex was validated and other subunits of this complex researchersre also identified in the IPs, including the histone deacetylases HDAC1 and HDAC2 [85]. The Upstream binding factor (UBF) is responsible for the recruitment of the RNA PolI pre-initiation complex required for rRNA transcription. It has been reported that deacetylation of UBF by HDAC1 disrupts the recruitment of UBF to PolI and causes a decrease in rDNA transcription, thus affecting cell proliferation [86]. In the prostate cancer cell line PC-3, silencing of the HMGB1 gene induced downregulation of key regulators of ribosome biogenesis and RNA processing such as OP1, RSS1, UBF1, KRR1, and LYAR. The analysis carried out using results from databases revealed that upregulation of these genes in prostate adenocarcinomas correlates with worse prognosis, reinforcing their functional significance in cancer progression [85].

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