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Yeast cell model in anticancer drug discovery

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Humanized yeast: in the front line of anticancer drug discovery

In the last years, valuable data about the biology and pharmacology of cancer-related human proteins have been provided by our research group using yeast. Furthermore, new targets and pharmacological regulators of these human proteins were identified. Also, new yeast target-based screening assays were developed, which revealed to be powerful tools in the discovery of new therapeutic opportunities against cancer.

1. Yeast as a cell model to study human proteins

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The high degree of conservation of cellular processes and molecular pathways with human cells has made the yeast *Saccharomyces cerevisiae* a powerful cell model to study human proteins and the molecular mechanisms underlying the pathobiology of several human diseases. In fact, the yeast cell system presents many advantages over other cell models, particularly: a) easy and low-cost manipulation and short generation time; b) well-defined genetic system and high amenability to genetic modifications; c) possibility for an independent analysis of a wide range of human proteins (or simplified networks of human proteins) without orthologues in this microorganism. In spite of this, it must be noted that as a unicellular organism several aspects of human disorders that rely on multicellularity and cell-cell interactions are difficult to evaluate. Moreover, as a simplified cell system, some genes involved in the pathobiology of the human disease may not be present in the yeast genome [reviewed in (Pereira *et al.*, 2012a,b)]. Still, although the obvious limitations of using yeast to study human disorders, when used in complementarity with higher eukaryotic cell systems, yeast may greatly contribute to the uncovering of the pathobiology of human disorders, as well as to the discovery of new therapeutic opportunities.

If the gene implicated in the human disease is conserved in yeast, its function can be directly studied in this organism. However, if the gene implicated in the human disease has no orthologues in yeast, its heterologous expression in this microorganism will allow the study of the human protein in a simpler eukaryotic environment, without the interference of other proteins with similar functions, endogenous regulators and redundant processes [reviewed in (Pereira *et al.*, 2012a,b)]. This so-called humanized yeast model has been widely used by our group for biological and pharmacological studies of human proteins with a key role in cancer progression.

2. Yeast as a target-based screening approach

Besides its crucial contribution to the clarification of several complex cellular processes, in recent years, yeast has emerged as a valuable tool in genetic and chemical large-scale screenings [reviewed in (Pereira *et al.*, 2012a,b)]. In fact, the yeast screening assays have been greatly useful in the first-line screening of potential active compounds to be tested in more complex cell models. The use of these assays in the early phase of the screening may greatly reduce the costs and may expedite the discovery of new therapeutic agents.

For long, yeast has been employed in drug discovery through the identification of compounds that cause a desirable physiological change, rather than modulate a specific protein. However, a limitation of this drug discovery strategy, which does not begin with a search against a specific target, is that the precise mechanism of action cannot be determined without first identifying the molecular target, which is frequently a costly and complex task. The possibility of reproducing the function of disease-associated human proteins in the cellular environment of this organism led to the

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development of target-specific assays, which have replaced the non-target-based strategy.

The most common procedure in yeast target-specific assays is the gene overexpression. This technique is quite popular in yeast and widely used by our group, consisting in the use of a high-copy library, in which a cDNA is expressed under the control of a regulatable promoter. In the last years, overexpression assays have been widely applied to human disease-associated proteins that induce a screenable phenotype, like growth arrest. In fact, most of the human proteins involved in cancer are toxic when overexpressed in yeast by interfering with growth-regulatory pathways (e.g. p53 and caspases). Based on this, the restoration of yeast cell growth is the basis of several chemical and genetic screenings for inhibitors of human target proteins. Likewise, the increase of the growth arrest induced by the foreign protein is the basis of several screenings for activators of human target proteins. These assays can be easily adapted to the high-throughput screening (HTS) based on simple measurements of the yeast cell growth by optical density. In fact, several works have already demonstrated the effectiveness of engineering yeast cells in the development of fast, selective and cost-effective HTS assays [reviewed in (Pereira *et al.*, 2012a,b)]. Despite this, other yeast target-based screening approaches have been proposed as complementary or even as an alternative to the cell growth systems. One example are those assays based on the use of reporter systems (e.g. *LacZ* and Luciferase), such as the yeast-based transactivation assays developed for the analysis of the p53 status in cancer cells.

3. Yeast as a valuable tool in anticancer drug research

During the last years, our research group has been focused on the development and validation of yeast target-based screening assays for distinct cancer-related human proteins, such as protein kinase C (PKC), caspases and p53 family proteins. With this approach, the pharmacological research directed to these key proteins has been performed [reviewed in (Gomez-Cassata *et al.*, 2012; Pereira *et al.*, 2012a,b; Silva *et al.* 2012)].

Concerning the PKC family proteins, several growth-inhibitory assays using yeast cells expressing individual isoforms of this family were developed. With these assays, the potency and isoform-selectivity of well-known PKC activators and inhibitors were characterized. Additionally, new PKC modulators were discovered (reviewed by Pereira *et al.*, 2012b; Silva *et al.* 2012), namely the diterpene compound 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U), a potent and selective activator of the novel PKC δ and ϵ with antiproliferative effects against several human tumour cell lines (Coutinho *et al.*, 2009a) [Portuguese Patent (Saraiva *et al.*, 2009)]. In this work, contrary to phorbol 12-myristate 13-acetate, which activates classical and novel PKCs inducing their translocation to the plasma membrane and a G2/M

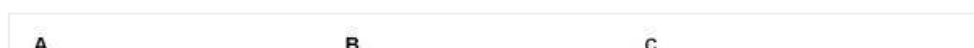
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cell cycle arrest, coleon U only activated the novel PKC δ and ϵ inducing their translocation to the nucleus and a metacaspase- and mitochondria-dependent apoptosis. Interestingly, these results confirmed that, as in mammalian cells, also in yeast distinct stimuli promoted the translocation of PKCs to different cellular compartments and subsequently the induction of distinct cellular responses.

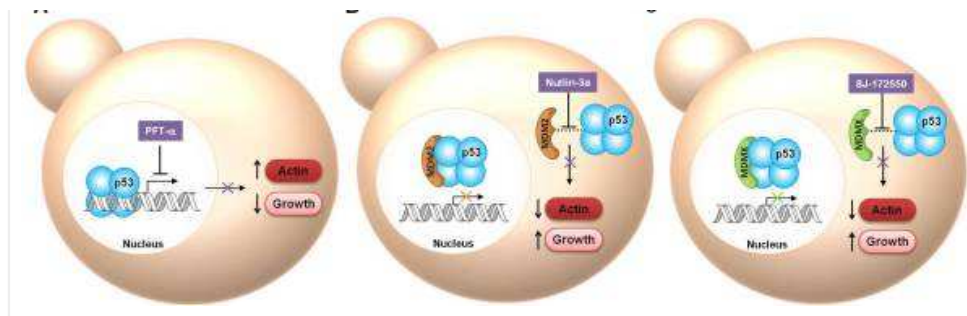
A similar growth-inhibitory assay to that of PKC isoforms was also developed for human caspase-3 and -7. In fact, using yeast cells individually expressing these caspases, new small molecule inhibitors of caspase-3 (Gloria *et al.*, 2011) and activators of caspase-7 (Pinto *et al.*, 2012) were identified from the screening of large libraries of aspartic vinyl sulfones and flavonoids, respectively. The identified compounds paved the way for a new class of small molecule modulators of caspase-3 and -7 with promising anticancer properties.

More recently, yeast has been used to study molecular and pharmacological aspects of human p53 regulatory pathway. The p53 tumour suppressor protein is a potent transcription factor with a crucial role in DNA repair, senescence, apoptosis, and cell cycle arrest, among others. The role of p53 as a gatekeeper of tumour suppression coupled to its great propensity to be a target for inactivation in tumours has made it a tempting choice for targeted therapy. In fact, the p53 activity is ubiquitously lost in cancers either by mutation or by inactivation of its protein due to the overexpression of negative regulators, namely MDM2 and MDMX. For these tumours, the reactivation of p53 activity may be possible by disrupting the interaction between MDM2 or MDMX with p53. Despite being structurally related proteins with the ability to inhibit the p53 transcriptional activity, evidence has emerged showing that these two proteins play multifaceted and non-redundant roles in modulating p53. Therefore, the identification of inhibitors of p53-MDM2 and p53-MDMX interaction has emerged as a promising therapeutic strategy against tumours retaining a wild-type p53 [reviewed in (Pei *et al.*, 2012; Shadfan *et al.*, 2012; Wade *et al.*, 2013)]. The development of new screening approaches that may improve and expedite this search will certainly contribute to achieve such a goal.

In our first work with p53, it was confirmed that expression of wild-type p53 in yeast induced a marked growth inhibition associated with S-phase cell cycle arrest. This toxic effect of p53 in yeast allowed the development of a yeast phenotypic assay, based on simple measurement of cell growth (Figure 1A). Using this approach, PKC δ and ϵ were identified as two natural activators of p53 transcription-dependent and -independent activity (Coutinho *et al.*, 2011). Additionally, this yeast growth-inhibitory assay was used as a screening tool to search for modulators of p53 activity. In subsequent studies, it was shown the conservation in yeast of the inhibitory effect of MDM2 and MDMX on p53 activity. In fact, as in mammalian cells, both MDM2 (Leão *et al.*, 2013a) and MDMX (Leão *et al.*, 2013c) inhibited the p53-induced yeast growth inhibition and cell cycle arrest.



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- Figure 1. Novel yeast growth-inhibitory and transactivation assays for p53 and p53-MDM2/MDMX interaction. Yeast growth-inhibitory assays are based on simple measurements of cell growth by optical density or colony-forming unit counts; yeast transactivation assays are based on the quantification of actin protein levels. (A) Yeast p53 assays. Expression of wild-type p53 in yeast induces growth inhibition and increases the actin protein levels; p53 effects are significantly reduced by pifithrin (PFT)- α , a selective inhibitor of p53 transcriptional activity. (B) Yeast p53-MDM2 assays. Co-expression of MDM2 inhibited the p53 effects (growth inhibition and increase of actin protein levels) in yeast; the negative effect of MDM2 on p53 activity is reverted by nutlin-3a, an inhibitor of the p53-MDM2 interaction. (C) Yeast p53-MDMX assays. Co-expression of MDMX inhibited the p53 effects (growth inhibition and increase of actin protein levels) in yeast; the negative effect of MDMX on p53 activity is reverted by SJ-172550, an inhibitor of the p53-MDMX interaction.

With the reconstitution in yeast of human p53-MDM2 and p53-MDMX networks, new simplified and cost-effective yeast growth screening assays to search for inhibitors of p53-MDM2 and p53-MDMX interaction, respectively, were developed (Leão *et al.*, 2013a,c) (Figure 1B, C). The use of the yeast p53-MDM2 interaction assay (Figure 1B), led us to the discovery of a new small molecule inhibitor of p53-MDM2 interaction with a xanthone scaffold (LEM1) (Leão *et al.*, 2013a), [International Patent (Inga *et al.*, 2013)]. Afterwards, by using the same yeast approach, two other xanthone derivatives with potent cytotoxic activity against several human tumour cells, a-mangostin and gambogic acid, were also identified as inhibitors of p53-MDM2 interaction (Leão *et al.*, 2013b).

Additionally, several authors have used the yeast p53 transactivation assay to study the impact of mutations, cofactors and small molecules on p53 transcriptional activity. These assays have been carried out using artificial yeast reporter constructs with response elements derived from human genes regulated by p53, such as *PUMA* and *BAX* [reviewed in (Pereira *et al.*, 2012b)]. Interestingly, in a recent work from our group (Leão *et al.*, 2013c), *ACT1* was identified as an endogenous p53 target gene in yeast. This finding opened the way to the development of a simplified yeast p53 transactivation assay, not based on artificial reporter constructs but on the quantification of actin expression levels for the analysis of p53 transcriptional activity (Figure 1A).

4. Final remarks

With the high number of potential therapeutic targets, as well as chemical libraries available to be tested, the development of fast, reliable and cost-effective yeast drug screening assays directed to target human proteins have been greatly useful in the early phase of the drug discovery process for the identification of potential active compounds to be tested in more complex cell models. In fact, the use of this yeast screening approach led us to the identification of new molecular probes and therapeutic opportunities against cancer.

We believe that relevant insights will continue emerging from yeast, contributing in a joint effort with more complex cell systems to face the challenges of therapeutic discovery in cancer.

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