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*Brief Communication*

# Use of tumor suppressor genes of naked mole rats for human cancer treatment

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**Abstract:** Cancer not only has a significant prevalence in the human population, but is also leading cause of death in animals. Despite a long history, our battle against cancer continues. Cross-species comparative genomics offers insight into shared genes and pathways by analyzing genomic data across species, enhancing our understanding of cancer mechanisms, evolutionary processes, and possible therapeutic targets. However, no previous study has demonstrated the inhibitory effects of tumor suppressor genes from one species on tumor cells from another. The naked mole rat is the only mammal yet to be found with cancer that is attributed to its tumor suppressor genes. In this study, we constructed phylogenetic trees and assessed the anti-tumor activity of two suppressor genes, programmed cell death molecule 5 (PDCD5) and dickkopf 3 (DKK3), from rats, mice, and humans. DKK3 robustly inhibited the proliferation of breast cancer cells within and across species due to its highly conserved protein sequence. However, the cross-species inhibitory effect of PDCD5 on breast cancer cells was inconsistent due to significant sequence variations. Intriguingly, PDCD5 from the naked mole rat demonstrated potent anti-tumor activity against breast cancer cells from mice, rats, and humans, surpassing that of PDCD5 from parental species. Our results demonstrate that the suppressor genes from the naked mole rat have a strong inhibitory effect on cancer cells, indicating that the powerful anti-cancer functions of the naked mole rat may be useful for human tumor treatment.

**Keywords:** Evolution, cancer, cross species comparison, tumor suppressor gene, target therapy

## Introduction

Cancer is a neoplasm formed by the overproliferation or abnormal differentiation of developing or mature cells driven by various carcinogenic factors [1]. Historical records of cancer date back to 3000-1500 BC, yet it remains a serious health threat today [2]. While cancer primarily affects humans, it also causes significant mortality in animals [3]. Tumor treatment methods have evolved, with contemporary approaches including surgical treatment, radiotherapy, chemotherapy, targeted therapy, and immunotherapy [4]. Of these, targeted therapy stands out as a particularly promising avenue in tumor therapeutics [5]. However, the genetic heterogeneity of tumors often leads to resistance against targeted treatments [5], making enhancing sensitivity to these therapies a pressing research priority.

Conserved gene mutations, whether within or across species, play a pivotal role in tumor development [6]. Cross-species tumor genomics is instrumental in identifying tumor suppressor genes [7]. Examining the various inhibitory effects of tumor suppressor genes across species can elucidate interspecies biological differences and offer deeper insight into tumor suppressor gene functions and carcinogenesis [7]. Through such comparative analysis, we can further develop a deep understanding of factors influencing tumor occurrence and development and establish a theoretical and scientific basis for personalized precision treatment [8].

In this study, we investigate the anti-cancer efficacy of two suppressor genes, PDCD5 and DKK3, across and within humans, mice, and rats. DKK3, a vital extracellular inhibitor of the classical Wnt signaling pathway, is highly con-

served throughout vertebrate evolution and often downregulated in human malignancies [9]. PDCD5, also known as TF-1 cell apoptosis-related gene 19, is frequently downregulated in various tumors, including breast cancer; it mediates apoptosis and impedes cancer cell migration by interactions with the apoptosis-activating factor, Apaf-1 [10].

Cancer resistance and susceptibility varies across species due to evolutionary natural selection [11]. Notably, the naked mole rat exhibits remarkable cancer resistance, attributed to its plethora of tumor suppressor genes and an absence of oncogenes [12]. Their efficient DNA repair mechanism prevents cumulative mutations during DNA replication, safeguarding against age-related health decline [12]. Thus, in addition to comparing PDCD5 from humans, mice, and rats, we evaluated the impact of PDCD5 from the naked mole rat on breast cancer cells. Furthermore, we compared the alterations in signaling pathways and transcription factors centered around PDCD5 and DKK3 in human, mouse, and rat breast cancer cells.

## Materials and methods

*Evolution of PDCD5 and DKK3 family*

The Ensembl genome browser (https://www.ensembl.org/index.html), which supports research in comparative genomics, evolution, sequence variation, and transcriptional regulation [13], was utilized to construct the phylogenetic tree of PDCD5 and DKK3.

*Protein sequence alignment*

DKK3 and PDCD5 protein sequences were sourced in FASTA format from the National Center for Biotechnology Information (NCBI) protein sequence databases. The three-dimensional structures of DKK3 and PDCD5 proteins were predicted using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2) [14]. The visualization software PyMOL was used for DKK3 and PDCD5 protein sequence alignment and for displaying their three-dimensional structures.

*Cell culture*

Human breast cancer cell line MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). Rat breast cancer cell line SHZ-88 cells and mouse breast cancer cell line 4T1 cells were obtained from Procell Life Science & Technology company (Wuhan, China). Cells were maintained at 37°C in a 5% $CO_2$ incubator in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 IU/ml).

*PDCD5 plasmid construction and transfection*

pEGFP-C1-PDCD5, which contains the human PDCD5 sequence, was stored in our laboratory. cDNAs of rat, mouse, or naked mole rat PDCD5 were amplified by PCR, subsequently digested with the appropriate restriction enzymes, and ligated into pEGFP-C1. Transfection of plasmids into MCF7, 4T1, and SHZ-88 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

*Recombinant DKK3 protein treatment*

Cells were treated with recombinant human DKK3 (hDKK3, 200 ng/ml), rat DKK3 (rDKK3, 200 ng/ml) or mouse DKK3 (mDKK3, 200 ng/ml) (Creative BioMart, Shirley, NY) for 24 hours.

*MTT assay*

48 hours after seeding cells ($1 \times 10^3$ cells/well) onto 96-well plates, 20 µl of MTT solution was added to each well. Absorbance was measured at 570 nm after 4 h using a TECAN microplate reader (Tecan Trading AG, Switzerland) to assess cell viability.

*cDNA library construction*

Total RNA was extracted from cells using TRIzol (TaKaRa, Dalian, China) according to the manufacturer's protocol. The quality of the library was measured using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). Library sequencing was performed using the Illumina HiSeq 2500 platform (Illumina, San Diego, CA).

*Differentially expressed genes (DEGs) analysis*

DESeq was used for the analysis of DEGs by comparing cells post-PDCD5 transfection or DKK3 treatment. Genes were considered significantly expressed at false discovery rate (FDR) values < 0.05 and fold changes ≥ 2.

*Western blot*

Cellular proteins (30 μg) were separated using 8% SDS-PAGE and transferred to nitrocellulose (NC) filter membranes (Beyotime). Membranes were incubated in 5% milk for 2 h at room temperature, followed by an overnight incubation with the primary antibody at 4°C. After 24 h, membranes were incubated with secondary antibodies for 2 h at room temperature. Staining was detected using an enhanced chemiluminescence kit (Beyotime).

*Statistical analysis*

Data are expressed as mean ± SD of three independent experiments, each conducted in triplicate. Differences between groups were determined by unpaired, two-tailed Student's t-test. A *P*-value < 0.05 was considered significant.

## Results

*Phylogroups evolve with different gain/loss rates of DKK3 or PDCD5 families*

The obtained phylogenetic tree, along with a matrix containing the number of homologous genes per protein family for each representative genome, were used to measure family sizes and lineage-specific events using an optimized gain-loss-duplicated model. Variability in gene content revealed that different phylogroups evolved with unique gain/loss/duplication rates of protein families. The assessment of DKK3 and PDCD5 copy numbers across species is presented in a gain/loss phylogenetic tree (**Figure 1A** and **1B**). DKK3 is highly conserved, whereas PDCD5 displays more mutations across species (**Figure 1C** and **1D**). Due to these variations in primary structure, there are notable differences in the quaternary structure of PDCD5 (**Figure 1D**).

*Differences in anticancer effects of DKK3 or PDCD5 from different species and genera*

The MTT assay showed that DKK3 from mice, rats, and humans can inhibit the proliferation of breast cancer cells from other species ($P < 0.05$, **Figure 2A**). DKK3 markedly influenced the proliferation of breast cancer cells within its own species ($P < 0.05$, **Figure 2A**). Due to the highly conservative sequence of DKK3 protein, there was no significant difference in the inhibitory rate of DKK3 on the proliferation of the three breast cancer cell lines (**Figure 2A**). Western blotting demonstrated that post DKK3 treatment, the levels of β-catenin, p-β-catenin, p-AKT, and p-mTOR were decreased in breast cancer cells, irrespective of species origin ($P < 0.05$, **Figure 2B**). RNA-sequencing showed that stemness regulating factors, SOX2, CMYC, Oct4, Klf4, Nanog, Lin28, Bmi1, Nestin, and FOXP3, and epithelial-mesenchymal transition (EMT) genes, EpCAM, N-cadherin, and vimentin were inhibited in breast cancer cells after DKK3 treatment ($P < 0.05$, **Figure 2C**). PDCD5 exhibited inhibitory effects on breast cancer cells from both the origin species and across other species ($P < 0.05$, **Figure 3A**). However, due to significant differences in the PDCD5 sequence between species, the inhibitory effect of PDCD5 on breast cancer cells from other species was significantly weaker ($P < 0.05$, **Figure 3A**). After PDDC5 treatment from the origin species, the protein levels of β-catenin, p-β-catenin, p-AKT, and p-mTOR in breast cancer cells of each species was lower than that of cells treated with cross-species PDDC5 ($P < 0.05$, **Figure 3B**). RNA-sequencing revealed the downregulation of SOX2, CMYC, Oct4, Klf4, Nanog, Lin28, Bmi1, Nestin, FOXP3, EpCAM, N-cadherin, and vimentin in breast cancer cells after PDCD5 treatment ($P < 0.05$, **Figure 3C**). Interestingly, PDCD5 from naked mole rat showed a strong anti-tumor activity against breast cancer cells from mice, rats, and humans, surpassing even PDCD5 treatment derived from their native species ($P < 0.05$, **Figure 3**).

## Discussion

Cross-species comparisons are used to identify genes central to tumor formation and, consequently, to pinpoint molecules instrumental in the onset and progression of human cancer [7]. Such comparisons have been applied in studies on liver, colorectal, breast, and prostate cancer [15-18]. Sweet-Cordero et al. [16] identified the significance of kirsten rat sarcoma (KRAS) 2 gene mutations in human lung cancer development by comparing gene expression profiles of lung cancer in mice and humans. Zender et al. [17] discovered that the apoptosis inhibitory gene, cellular inhibitors of apoptosis 1 (cIAP-1), and the transcription factor, yes-associated protein (YAP), positioned on human chromosome 11q22-corresponding to the same location on mouse chromosome 9qA1-promoted liver cancer development. In addition to tumors in mice, liver tumors in rats and zebraf-

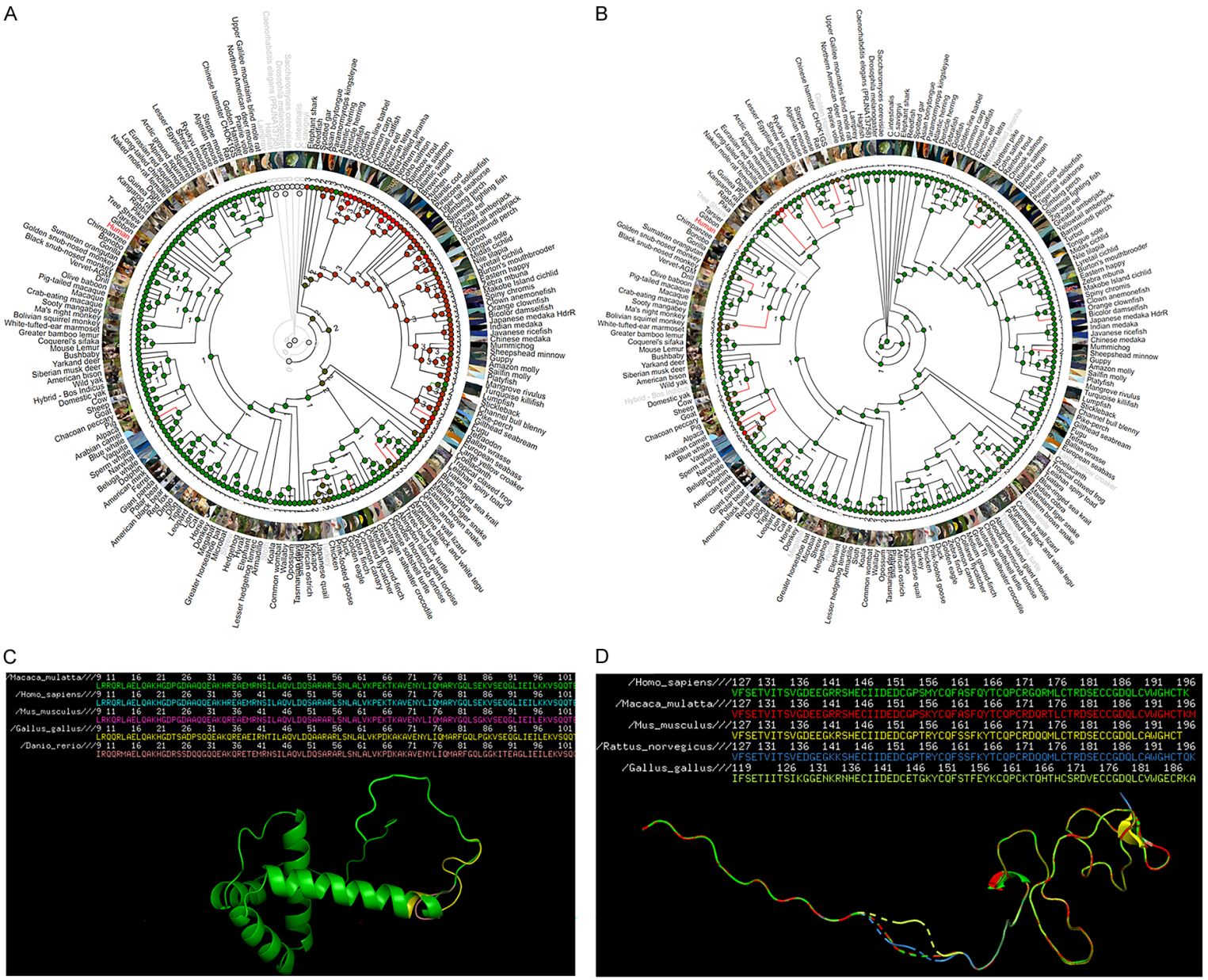


**Figure 1.** Phylogenetic gain/loss trees of DKK3 gene (A) and PDCD5 gene (B) were created in www.ensembl.com. The trees showed the number of DKK3 and PDCD5 paralogues/copies for each species or taxon. Red lines mark significant expansion of the DKK3 and PDCD5 gene copies. Comparison of protein sequence and 3D structure of DKK3 (C) and PDCD5 (D) using the visualization software PyMOL.

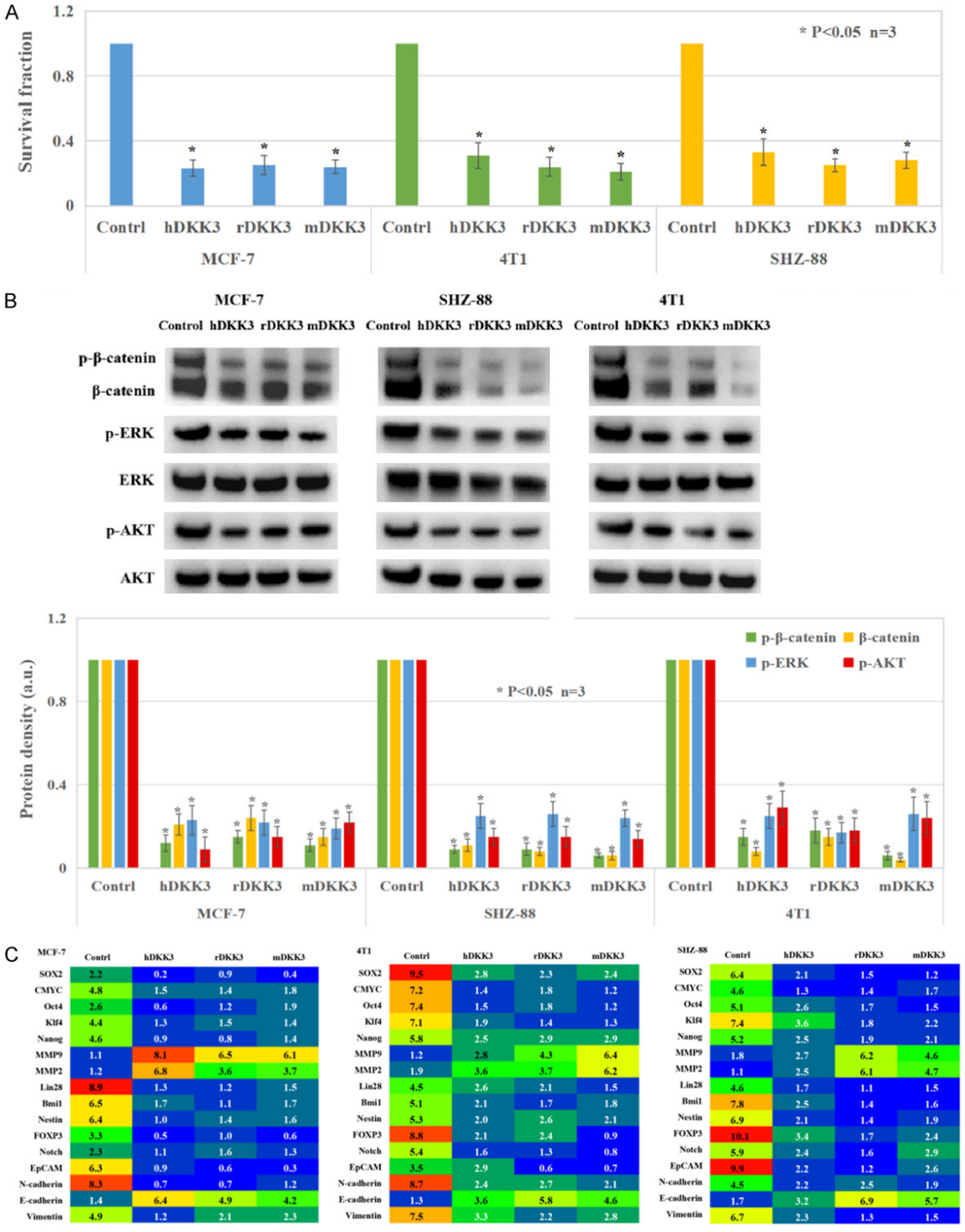


**Figure 2.** Anti-tumor effect and molecular mechanisms of DKK3 on breast cancer cells. A. Proliferation of human, rat, and mouse breast cancer cells with DKK3 treatment was measured using the MTT assay. B. Changes in β-catenin, p-β-catenin, p-AKT and p-mTOR in DKK3-treated breast cancer cells and their parental cells were studied using western blotting. C. Stemness regulatory transcription factors, SOX2, Nanog, C-myc and Oct4, in DKK3-treated breast cancer cells and their parental cells were studied using RNA sequencing.

ish were found to closely resemble human liver tumors, suggesting that conserved molecular changes across species may play a critical role in liver tumor progression [18]. By studying

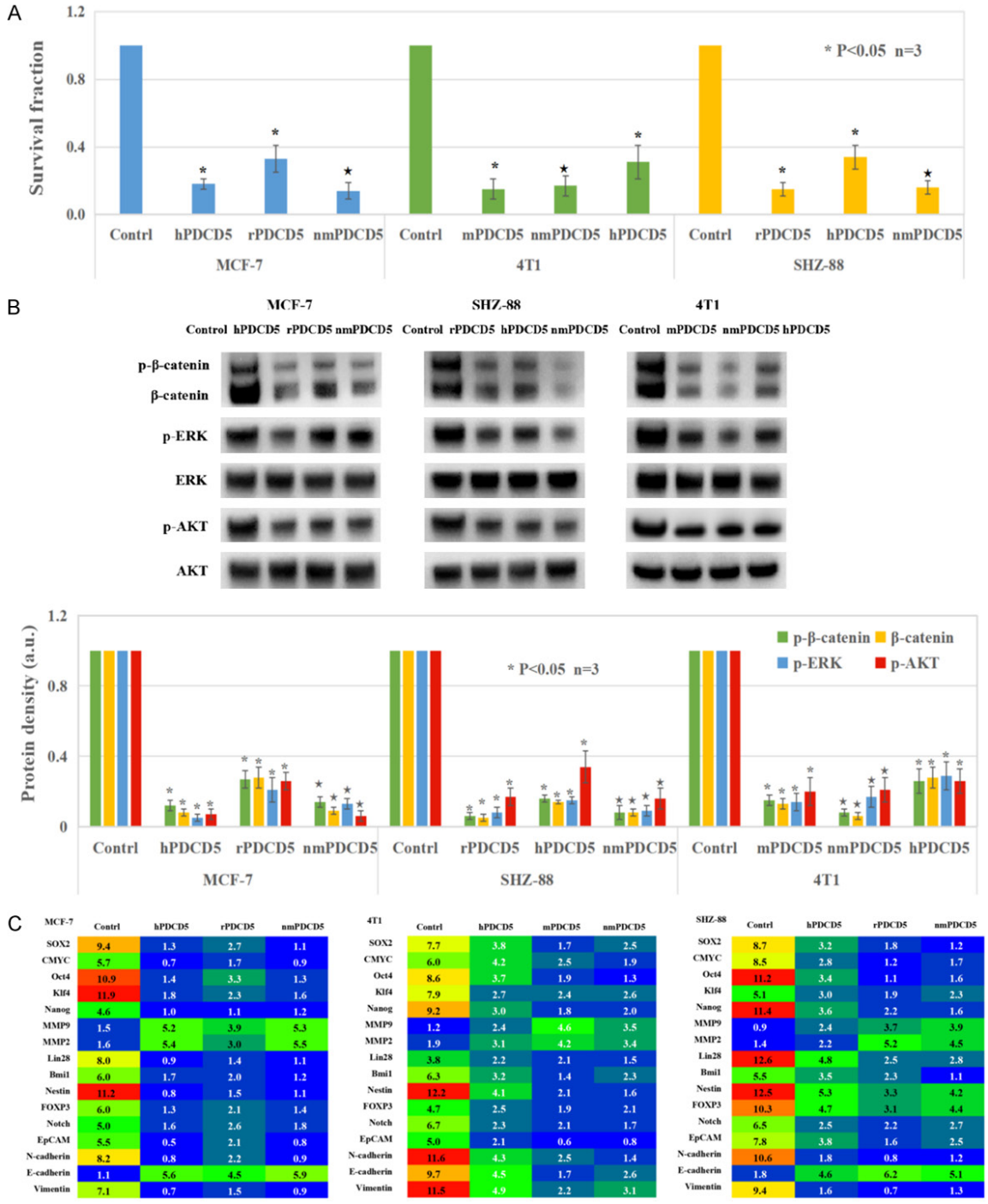


**Figure 3.** Anti-tumor effect and molecular mechanisms of PDCD5 on breast cancer cells. A. Proliferation of human, rat, and mouse breast cancer cells with DKK3 treatment was measured using the MTT assay. B. Changes of β-catenin, p-β-catenin, p-AKT, and p-mTOR in PDCD5-treated breast cancer cells and their parental cells were studied using western blotting. C. Stemness regulatory transcription factors, SOX2, Nanog, C-myc and Oct4, in PDCD5-treated breast cancer cells and their parental cells were studied using RNA sequencing.

cross-species anti-tumor strategies, we can gain a deeper understanding into the underlying mechanism of tumor occurrence, evolution, and gene regulation, providing novel avenues and methods for exploring the mechanisms of tumor occurrence and treatment. Due to genetic mutations, human tumor suppressor genes and their encoded products might not be the

strongest compared to homologous genes in other animals. To our knowledge, no studies have contrasted the inhibitory effects of cancer suppressor genes across diverse species and genera. In this study, we found no significant differences in the anti-tumor effect of DKK3 across species. The driving mechanism is that DKK3 is highly conserved among various species. Notably, DKK3 from the same species demonstrated the highest inhibitory effect on its native species. Conversely, PDCD5, another tumor suppressor gene, did not display strong anti-tumor effects across species. Juxtaposition of PDCD5 protein quaternary structure across species revealed significant changes to protein structure driven by changes in primary structure.

A pivotal discovery in our study was the pronounced anti-tumor activity of PDCD5 from naked mole rats in rat, mouse, and human cancer cells. The susceptibility to cancer varies among mammals, influenced in part by dietary habits [11]. Predatory animals, particularly apex predators like clouded leopards and red wolves, face heightened cancer risks, with over 25% succumbing to the disease [11]. Compared to deep-sea fish, livestock such as chickens, ducks, pigs, cattle, and sheep have a substantially higher cancer incidence [11]. This is attributed to their shared environments with humans, providing daily exposure to and potential ingestion of carcinogens. These observations highlight that prolonged environmental exposures can influence the evolution of distinct anticancer mechanisms. Naked mole rats are currently the only mammal that can survive under low and anaerobic conditions, possessing extraordinary anti-cancer capabilities [13]. There have been no documented cases of cancer in naked mole rats, a phenomenon partly attributed to their abundant high molecular weight hyaluronic acid [13]. However, past research has not explored leveraging the formidable anti-cancer potential of naked mole rats in human cancer therapy. Our study established that the PDCD5 gene from the naked mole rat exhibited potent anti-tumor effects against cells across species, surpassing even the native PDCD5 gene of parental species. Consistent to previous studies, we also verified that both DKK3 and PDCD5 inhibit the AKT/mTOR and Wnt/β-catenin signaling pathways. Furthermore, stem cell markers, including SOX2, Nanog, KLF4, and OCT4, were also inhibited by DKK3 and PDCD5 from humans, mice, and rats-most notably, PDCD5 from the naked mole rat.

## Conclusion

In this study, we confirmed DNA sequence conservation determined the anti-tumor activity of tumor suppressor genes across species. The tumor suppressor genes of each species exert the strongest tumor suppressor effect on their respective species. The tumor suppressor genes from the naked mole rat also display robust tumor-suppressive effects on other species, introducing novel avenues for human cancer treatment. Nonetheless, *in vivo* validation of our findings will be crucial, especially to preclude allergic reactions in patients.

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## Disclosure of conflict of interest

None.

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