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CREATION OF U251 SENSING CELL LINES THROUGH LIPOFECTION WITH NUCLEUS AND CYTOPLASM HAVING DIFFERENT OPTICAL SIGNATURES

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ABSTRACT
The bioactivity of new drugs may result in morphological alterations at the single cell level that are indicative of cell vitality. With current technology permits the detection of perturbations in the cell cycle, such as mitosis, senescence or death, using non-invasive methods in live cells. Our objective is to develop sensor lines for the real time visualization of changes in chromatin or cytoplasmic morphology. A palate of optical markers such as fusion proteins that contain histone H2B and a fluorescent protein, such as Katushka would reveal nuclear alterations. Changes in the cytoplasm can be revealed using a fusion between actin and CFP. For example, a cell entering senescence could be identified by distinct optical signatures such as weakly stained, relaxed chromatin and an enlarged, flattened cytoplasm. A cell in mitosis would be characterized by condensed chromatin and the particular phase could be deduced from the chromosomal arrangement. Using plasmid vectors we have shown that such assays are reliable. The cDNAs encoding these fusion proteins are being transferred to cells with lipofection, These tools could permit the detection of morphologic changes in single cells by time-lapse microscopy.

KEYWORDS: Katushka, Sensing Cells, fluorescent proteins.

INTRODUCTION
Catalytic complexes formed between the association of cyclin-dependent protein kinase (CDK) with a cyclin (positive regulatory subunit) are responsible for cell division. The control of the cell cycle is done by consecutive arrangements and disarrangements between protein kinases and cyclins, commanded by the pRB-E2F-p16^{INK4a} and p53-p21^{CiPl} -p14^{ARF}-MDM2 pathways, which are responsible for controlling DNA replication and checkpoints) GI/S and G2/M. CDK4/6, CDK2 and CDKI or cdc2 associate respectively with cyclins D, E, A or B, promoting the phosphorylation of pRb and release of transcription factors E2Fs that result in different stages of the cell (Sawin, 2009; Strauss et al., 2005).

Population analysis of cells by flow cytometry is possible, this technology allows us to observe in which cellular stages (Gl, S, G2) are found a set of cells labeled with fluorescent proteins or stained with propidium iodide (Conquelle et al., 2006; Sakawe-Sawano, 2008; Beaves & Kalejta, 1999; Harvey et al., 2001). Currently, there are a wide range of fluorescent proteins in different wavelengths.

Since the invention of the light microscope, the cell has been studied. With the advent of binding technology with fluorescent molecules, it is now possible to study dynamics in living cells, for example, green fluorescent protein (GFP) has revolutionized cell imaging research, because auto-fluorescent molecules can be genetically fused with cDNA of interest (Stephens & Allan, 2003).

Green Fluorescent Protein (GFP) is a protein isolated from a gene of the jellyfish *Aequoria victoria*, first by Shimomura in 1962 with the purification of the bioluminescent protein aequorin. Morise and collaborators in 1974 carried out an assay which demonstrated the fluorescent properties of GFP with the emission of green light in the energy transfer of aequorin. In 1994 Chalfie and collaborators published an article in Science showing that GFP could be used as a marker for localization of proteins and expression in live bacteria and in worm cells, proving to be an excellent tool for the study protein and addressing (Paterson & Lippincott-Schwartz, 2002).

Later, the wild GFP gene underwent successive modifications, for example, the addition of the Kozak

motif resulted in the enhanced GFP (eGFP) version, optimized for cell expression in eukaryotes, with a 488nm excitation peak and a 507nm emission peak, which revolutionized imaging studies cell phones (Paterson & Lippincott-Schwartz, 2002).

Mutations replacing the amino acids Tyr66 by His66 or Trp66 enabled other spectral forms such as the BFP (Blue Fluorescent Protein) and CFP (Cian Fluorescent Protein) versions with absorption peaks of 384nm and 420nm respectively. Also the YFP (Yellow Fluorescent Protein) version, which is characterized by the point mutation Thr203Tyr and has excitation in the 514nm range. Another mutated protein derived from YFP is Citrine, which has an increase in yellow fluorescence intensity and greater acid resistance. (Fraser et. al, 2005; Griesbeck et al., 2001).

In addition to GFP and its variants, there are fluorescent proteins in the infrared light spectrum, the first red fluorescent protein was extracted from *Discosoma* sp. called dsRed. Today there is a vast diversity of variant proteins baptized with fruit names: mCherry, mBanana, tdTomato, mTangerine, mStrawberry and mPlum which occupy the ranges between orange and red yellow, are monomeric and tolerant to amino-terminal fusions and have fast rates of maturation (Shanner et al., 2005).

Red fluorescent proteins particularly hold promise in breeding transgenic animals. However, a common problem in many of these proteins is it cytotoxicity, which is why genetic engineers spend more time in their construction. Bevis & Glick et al. (2002), developed a variant of DsRed, the DsRed.T3 protein which decreases cytotoxicity in some cell types. In an experiment Vintersten et al. (2004) manage to transduce the gene of this protein into a mouse embryo, generating transgenic animals for DsRed.T3 (Vintersten et al., 2004; Davidson & Campbell, 2009).

There is yet another protein in the infrared range called Katushka, and its monomeric version mKate derived from the sea anemone *Entacmaea quadricolor*. This protein has rapid maturation at 37°C with a time of 20 min, which is faster than mcherry, approximately 40 min. High pH stability and high gloss (Shcherbo et al., 2007).

Another example of a red fluorescent protein is extracted from the *Fungia concinna* coral, called Kusabira Orange (KO) in Japanese Kusabira Ishi, which has low toxicity and high fluorescence. The gene for this protein was transfected with the retroviral vector pMSCV into hematopoietic stem cells to analyze the dynamics of these cells in mice (Sanuki et al. 2008).

Histones are the main structural proteins of chromatin and are ideal for labeling, for example, several works describe the fusion of proteins between Histone H2B and GFP, which allows visualization of the cell nucleus, with histone being a specifically nuclear protein. Thus, healthy cells could be used and manipulated in FACS (Fluorescence-activated cell sorter), and purified in different cycle phases such as Gl, S and G2. (Conquelle et al., 2006; Kanda et al, 1998).

Recently, several cell cycle markers specific for S and G2 phases have been created using the fusion of bioluminescent proteins with PCNA molecules. This is a key molecule in the DNA replication machinery involving the processes of genetic material elongation, recombination, methylation and repair. During PCNA synthesis it forms a sliding hairpin to DNA which recruits Polymerases (ô, e and a mitochondrial - 7) and displaces polymerase u. This central component of the DNA replication machinery is not only linked to polymerases but also ligase-1, helicases II, replication protein A (RPA), topoisomerases I and II. The fusion between GFP-PCNA remains fused in the nucleus and during the extension of the prophase it dissociates from the DNA. This strategy is very useful for studying the S phase of cell cycle progression in vivo. (Kisielewska et al., 2005; Essers et al., 2005; Leonhardt et al., 2000).

Still in this same context of study in the S phase of the cell cycle, studies describe a labeling with the DS Red protein fused with the protein ligase. And also the fusion between Dnml (DNA methyltransferase) with GFP. Since the activity of these proteins are different in the cell cycle, it is possible to verify the cell dynamics in different phases (Easwaran et al. 2005).

Another strategy used with fluorescent protein labeling is the Fucci system ("Fluorecent Ubiquitination-based cell-cycle indicator"). The Cdtl molecule is specifically accumulated during the GI phase of the cell cycle, but it is degraded during the GI/S transition after ubiquitination. Geminin is accumulated during S, G2 and M phase and degraded at the end of mitosis. Then the fusion of proteins Cdt1-mK02 (Kusabira Orange) and Geminin-mAG (Azami Green) is used, this allows a sensor cell which during the Gl phase is Orange and during the S/ G2/M phase is green (Sakawe- Sawano, 2008).

Certain mutated molecules of the DsRed protein, with substitutions Va1105 for Ala105 and Ser197 for Thr197, called E5, have the advantage of being a temporal fluorescent marker, that is, it changes its fluorescence from green to red over time. These proteins also have yellow and orange fluorescence, being intermediate between green and red, demonstrating that both fluorophores are present. E5 has the potential to function as a molecular clock giving spatiotemporal information to specific target promoters (Terskikh et al., 2000).

Other studies aimed at fluorescent protein fusion describe the construction of three protein fusion (MTF), red fluorecent protein (mRFP), Firefly Luciferase (FL) and HSVI sr39 truncated Thymidine Kinase (TK), which is linked to caspase-3 recognizes the polypeptide

binding site. After cleavage by Caspase-3 it gains significantly in mRFP, FL and TK activity. Thus, when the caspase enzyme comes into activity, it releases the fluorescence of the proteins which are fused (Ray et al., 2008).

Dynamic and in vivo imaging of the cell cycle is essential to determine the normal course of cell division and to follow the effects of natural or experimental disturbances. In this proposal, it is intended to visualize the cell cycle in vivo and in real time, hence the need for the construction of cell cycle sensor lines. Thus, it involves the strategy of using lipofection for nuclear (pTurboH2B-Kate) and cytoplasmic (pMSCVBFP-Actin) labeling. In vivo and real-time images of the chromatin-cytoplasm dynamics during division allow, for example, to analyze the relationship between nuclear and cytoplasmic volume, an important indicator of senescence and cell death.

MATERIAL AND METHODS

The construction of plasmid pTurboH2BKate followed cloning steps by recombinant DNA. Initially, the fusions between the human H2B gene and the fluorescent protein (PF) gene were made by cloning the H2B fragment into expression vectors carrying the PF genes: peGFP- Nl (Clontech, USA), pYFP-N1 (given by Dr. R. Tsien University of California, San Diego, USA) and pTurboFP635-N (Evrogen, Russia). This step is essential because it allows testing the functionality of the chimeric protein before transferring the H2B-FP cassette.

The human H2B gene (GenBank accession # X00088) was obtained by PCR from genomic DNA extracted from a primary culture of human fibroblasts (kindly donated by Dr. L. Isaac (Depto. de imunologia, ICB, USP) using the primers:
F:5'CGGGTACCGCCACCATGCCAGAGCCAGCGAA GTCTGCT 3' and R: 5' - CGGGATCCITAGCGCTGGTGTACITGGTGAC - 3'.

The PCR reactions were carried out in collaboration with the student Felipe Lager and were assembled using Platinum Taq DNA Polymerase with 100ng of DNA and 25picoMol of each primer. The following PCR reaction parameters were applied to amplify 400bp H2B fragment: 94^{0}C for 1 min., 50^{0}C for 1min. and 72^{0}C for 2 min., repeated for 25 cycles of PCR. This pair of primers served to introduce sites for the restriction enzymes Kpn I and Bam HI, respectively in the 5' and 3' portion of H2B. Primer F also served to introduce the sequence corresponding to the Kozak motif in front of the initiation codon. The PCR product, H2B, was purified using Concert Rapid PCR Purification Kit, Invitrogen, USA) and digested with the enzymes Kpn I and Bam HI (both supplied by Invitrogen, USA). After treatment with restriction endonucleases, the H2B/KpnI-BamHI fragment was purified with a phenol/chloroform mixture, precipitated in the presence of ethanol/NaCl and resuspended.

The expression vectors peGFP-N1 and pYFP-N1 were linearized using double digestion with the enzymes Kpn I and Bam HI, purified with a mixture of phenol/chloroform, precipitated in the presence of ethanol/NaCl and resuspended in water. Digested and purified samples of vectors and H2B/Kpnl-BamH1 PCR product were analyzed using gel electrophoresis (0.80/0 agarose/1X TAE) prior to binding reactions.

For the binding reactions, the enzyme T4 ligase (New England, USA) was used, respecting the 3:1 ratio between free ends of insert:vector. Chemically competent E. coli DH5α bacteria were transformed using 1/5-1/10 the volume of the ligation reaction. Plasmid DNA from kanamycin antibiotic resistant clones was extracted with PureLink Quick Plasmid Miniprep Kit (Invitrogen, USA). Plasmid DNAs from clones carrying the H2B-Kate chimeras were identified through restriction gel with the release of the 400bp H2B insert obtained after double digestion with Kpn I and Bam HI. Glycerol stocks from clones with pH2B-Kate identity were prepared and stored at -70^{0}C.

In the construction of vector pTurboH2B-FP635 (simply pTurboH2B-Kate) the H2B expression cassette was removed from vector pH2B-YFP and subcloned into vector pTurboFP635 (also known as pTurboKate), following the same protocols for digestion, binding and transformation of bacteria as described above (Fig1).

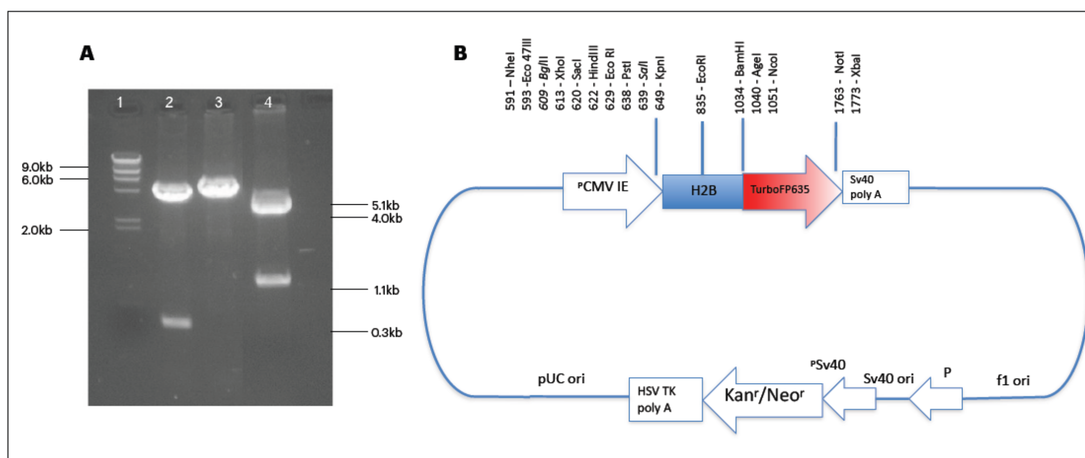

Fig1. Construction of the nuclear sensor expression vector carrying the H2B-Kate chimeric protein with infrared fluorescence. A: Cloning scheme of the H2B gene transferred by recombinant DNA technique from the pH2B-YFP vector to the pTurbo vector. (1) Lambda Hind III molecular weight marker (2) pTurbo-Kate double digestion with restriction enzymes BamHI and Kpnl releasing H2B fragment (0.3Kb) (3) Simple digestion with BaHI restriction enzyme linearizing pTurbo vector H2B-Kate. (4) Double digestion with restriction enzymes BamHI and NotI releasing the fragment of the fused genes H2B-Kate (1.1Kb). B: Restriction map of the nuclear sensor expression vector pTurboH2B-Kate.

Medium-scale preparations (Midi preps) of pTurboH2B-Kate vectors were obtained with the PureLink Quick Plasmid Midiprep Kit system (Invitrogen, USA) and stored at -20^{0}C. Plasmid DNA concentration was determined by spectrophotometry at 260nm and the 260nm/ 280nm ratio served as an indicator of purity and protein contamination of plasmid preparations, following the classic protocol (Sambrook, 1986).

Cell line maintenance

Cells of the U251 MG derived from human glioblastoma multiforme, were cultured with DMEM medium (Dulbecco's Modified Eagles' Medium, Invitrogen USA) supplemented with 100 mg/ml of gentamicin, 50 mg/ml ampicillin, 50 mg/ml fungizone and 10% fetal bovine serum (Hyclone, USA). All cultures were kept at 37^{0}C in a humid atmosphere with 5% CO_2. Placing and subcultures were performed after treatment of cultures with TripLE Express (Invitrogen, USA).

Lipofection and Selection

The transduction method was lipofection (liposome transfection), using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's protocol. Approximately cells/ well were plated in 24-well tray. After 24 hours the original culture medium from the plating (antibiotic-free DMEM with 10% fetal bovine serum) was replaced by 500 µl of the Opti-MEM medium (Invitrogen, USA). Two solutions, one containing 0.8 µg of each expression vector of the pH2B-FP or pTagCFP-actin series (Evrogen, Russia) and 50 µl of Opti-MEM and the other containing 2 µl of Lipofectamine 2000 (Invitrogen, USA) dissolved in 50 µl of Opti-MEM were mixed and incubated for 30 min. and immediately added to each well. Twenty-four hours after lipofection, the culture medium was replaced by DMEM supplemented with 600µg/ml of the antibiotic G418 (Invitrogen, USA). Between 7-10 days after the start of selection with G418, all cells from the control plate (without DNA) died, indicating the end of selection. The cells were kept under selection for another 7-10 days, expanded and samples of the new sensor lines expressing the chimeric proteins pH2B-Kate and pTagCFP-actin were frozen and stored in liquid nitrogen Dual U251 cell transfectants were obtained with the vectors pTurboH2B-Kate and pTagCFP-Actin following the lipofection and selection protocol described above.

Fluorescence Microscopy

Images were captured directly from cells in culture (live) with the aid of a confocal microscope (Axiovert Zeiss, Germany) installed at the Department. of Physiology and Biophysics, ICB.USP. In this assay about 5 X 105 cells from each clone were plated on P35 plates and the fluorescence of the H2B-Kate chimera proteins was visualized 48 hours after plating. The laser was adjusted for excitation/emission in the ranges between 588-633, respectively compatible for detection of Kate protein fluorescence and panoramic images captured with a 40X objective. For better identification of mitosis figures, images were also captured under an immersion objective from cells cultivated on coverslips. Briefly, around 106 cells of the HT1080pTurboH2b-Kate strains were plated on coverslips placed on the bottom of P35 plates, 48h. after plating, the culture medium was removed, the cells washed with PBS IX and fixed with 0.5% glutaraldehyde for 10 minutes at 40°C. The fixative was removed, the coverslips washed with PBS 1X and slides were mounted with Vectashield (Vector Laboratories, USA). The preparations were observed under confocal microscopy with a 100X immersion objective and the captured images edited in the LSM Image Browser program.

RESULTS

Nuclear localization of H2B-FP chimera proteins

Figure 2 shows images of the U251pTurbo-Kate sensor lines obtained from living cells in culture. We can observe the nuclear tag, confirming the location of the chimera fluorescent protein in chromatin. Again, these images show a heterogeneous population of cells, although all cells are resistant to the antibiotic G418 it can be seen that not all resistant cells express the H2B-FP proteins. This is an expected result due to the non-viral gene transfer system used in this trial.

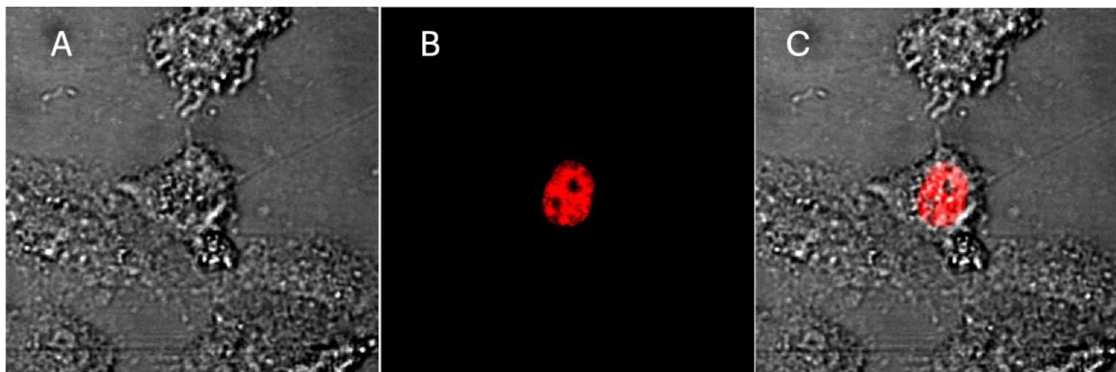

Fig2. Confocal microscopy images evidencing PTurbo-Kate U251 sensor lines obtained from living cells in culture. (A) Cells panel of photos captured in bright field. (B) Cells panel of photos captured in dark field (588nm). (C) A and B overlaid.

Dual Lipofection of the Nucleus and Cytoplasm

In an alternative of simultaneous visualization of joint nuclear and cytoplasmic labeling, U251 cells were co-lipofected with the expression vectors pTurboH2B-kate and pTagCFP-actin (following the same lipofection protocol). These can be seen in Fig. 3, which respectively confer the red nuclear and blue cytoplasmic optical signature (aquamarine).

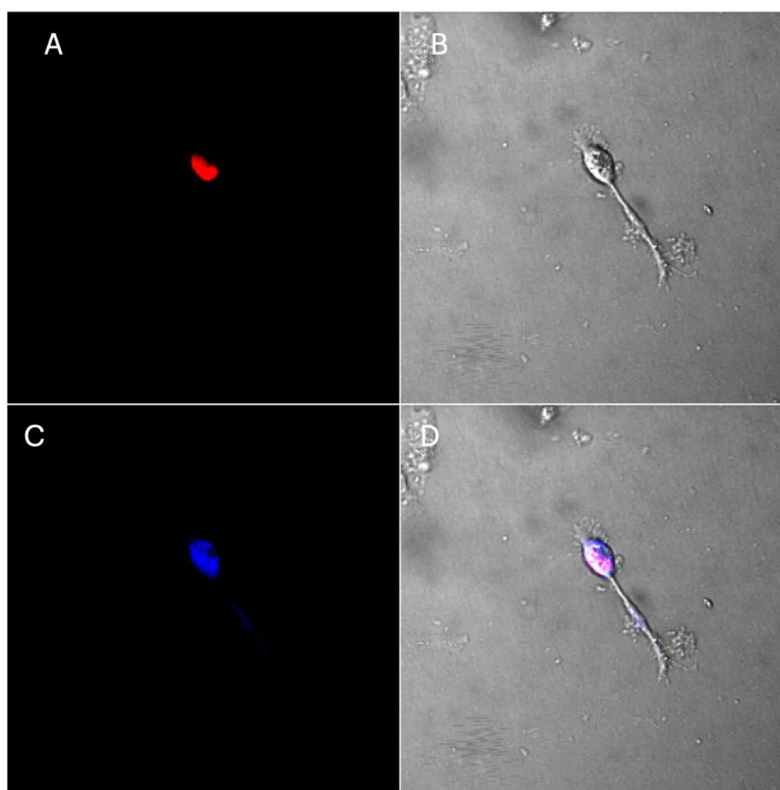

Fig3. Image of U251 cells expressing nuclear and cytoplasmic labeling doubly transfected with pTurboH2B-Kate and pTagCFP-Actin sensor vectors obtained from fixed cell preparation. 1000X Immersion Objective. (A) Darkfield images revealing the nuclear expression of the 388nm H2B-Kate fusion. (B) Image of U251 cell viewed in bright field. (C) Darkfield images revealing cytoplasmic expression of the CFP-Actin fusion captured at 439nm. (D) Overlay of images A, B and C.

DISCUSSION

Through lipofection and selection assays to evaluate the functionality of the vectors pTurboH2B-Kate and pTagCFP-Actin, they were shown to be effective in marking both the nucleus and the cytoplasm of U25l Cells.

Cells with incorporation of genes fused with fluorescent proteins are not toxic to cells and do not alter the cell cycle dynamics. Thus, the use of fluorescent proteins fused to genes has become promising for the study of cell biology, even being able to film a sensor cell in its cycle.

This strategy allows determining the position of the cell cycle and has several advantages: 1 - When anchored to a nuclear structure, it concentrates and increases the fluorescent signal. 2 - It remains attached to the nucleosome throughout cell division, even when the nuclear envelope is undone. 3- Follows the phases of mitosis without cell destruction. 4 - It is resistant to treatment with fixatives and permeabilizing agents. 5 - Clearly distinguishes interphase and mitotic nuclei. 6 - It is not toxic and does not affect cell cycle progression (Essers et al., 2005).

Since plasmid vectors do not integrate into the cell, but only in episomal form, one strategy would be to clone the cassettes of interest into retroviral vectors, thus transduction with them would become more effective as they integrate into the host cell's genome, permanently marking and creating an optical signature the sensor cells.

Conquelle et al., (2006) in their article tests cells as sensors to assess the susceptibility of phases G1, S, G2 with cytotoxic drugs, such as stauroporine, camptotenin and BAY117082, in this trial 2% of the population died spontaneously from points, however some were successive to cell death and had growth inhibition as an effect of the drugs.

CONCLUSION

The creation of sensor lines would be a great mechanism to assess cell cycle dynamics, for example in sensor tumor cells treated with tumor suppressor genes or chemotherapy drugs for the treatment of cancer. Also including the evaluation of new drugs to be used as pharmaceuticals in a growing industry.

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