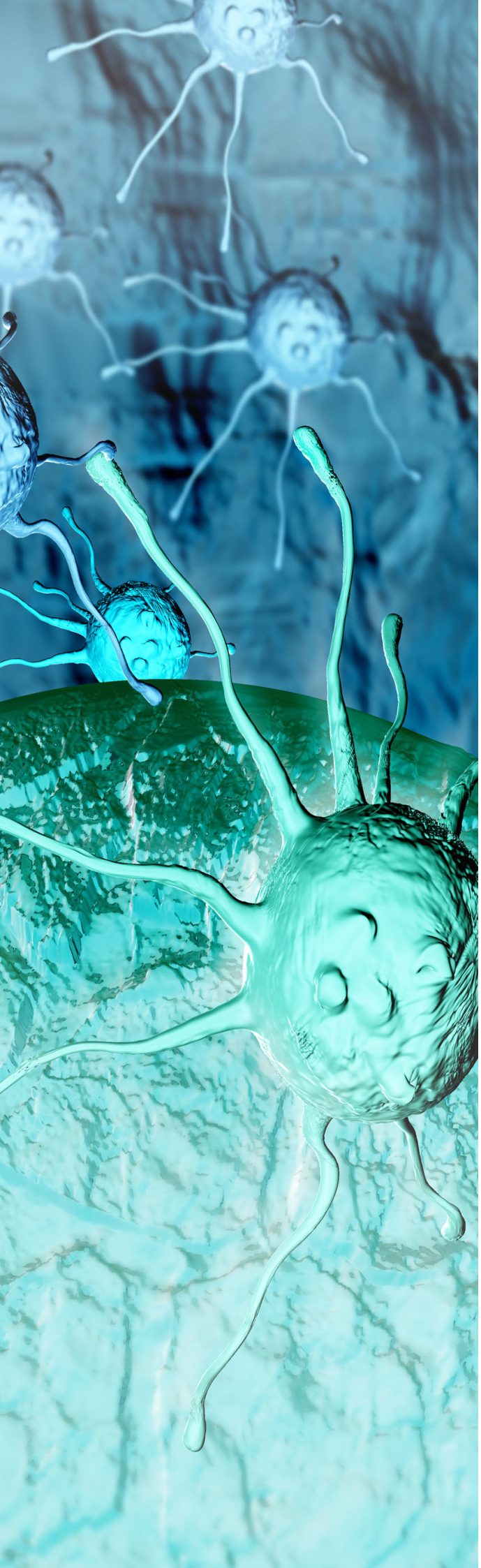

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Corresponding author
Pradeep K. Garg, PhD
Executive Director
Center for Molecular Imaging and Therapy, Biomedical Research Foundation of NW Louisiana
Shreveport, LA 71106, USA
Tel: 318-675-4002
E-mail: pgarg@biomed.org

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Role of Molecular Imaging in Oncology

Pradeep K. Garg[1*], Surinder K. Batra[2] and Sudha Garg[1]

[1]Center for Molecular Imaging and Therapy, Biomedical Research Foundation of NW Louisiana, Shreveport, LA 71133, USA

[2]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198, USA

ABSTRACT

Molecular Imaging (MI) is an emerging technology for the early detection of disease, staging of the disease, and for monitoring response to therapy. It also offers a non-invasive method to detect *in vivo* biological functions and processes at a molecular level. The use of MI requires careful selection of targeting molecules which are expressed differentially in diseased *vs.* healthy cells to interrogate the cell microenvironment. Targeting molecules for MI could consist of small molecules, single amino acid units, low molecular weight peptides, antibodies or antibody fragments. Over the years, a large number of small molecular weight imaging probes have been developed to target different molecular pathways using Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). The use of high molecular weight probes such as radioactive antibodies (Ab) is also equally attractive. While a major effort is placed on developing radioactive probes for PET and SPECT imaging, an intense effort is being focused on enhancing the utility of other MI modalities such as nuclear Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS), Computed Tomography (CT), optical imaging and ultrasound (US).

KEYWORDS: Antibody; Imaging; SPECT; PET; Tumor; Optical.

INTRODUCTION

Molecular Imaging (MI) is an emerging field that combines the non-invasive monitoring of *in vivo* biological processes at the cellular level and the anatomic information associated with tissues undergoing such transformations. MI therefore has evolved as an indispensable technique for the early detection and diseasestate, and for monitoring response to therapy. These modalities offer non-invasive detection of *in vivo* biological functions and processes at the molecular level through the careful selection of targeting molecules which is expressed differentially in diseased *vs.* healthy cells to interrogate the cell microenvironment. These targeting molecules could consist of small molecules, single amino acid units, low molecular weight peptides, antibodies or antibody fragments. There are now a large array of imaging technologies under the MI umbrella which include Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Computed Tomography (CT), ultrasound (US), bioluminescence (quantum dots), and fluorescence imaging (optical imaging).[1] Amongst the tools required for targeting diseased tissues to obtain clinically relevant information, the key is to match the optimal targeting molecule with the imaging modality of choice.

RESULTS AND DISCUSSIONS

Amongst various options within MI, nuclear imaging is the more common non-invasive detection technique to target tumors. Nuclear imaging employs radiolabeled tracers which concentrate in cancerous tissues and generates the radioactive signal for visualization of tu-

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-mors. This signal is detected, reconstructed, and analyzed with the resulting image corresponding to the spatial distribution of the radiotracer in the cancer mass and surrounding tissues. The two major nuclear imaging modalities are SPECT and PET.

POSITRON EMISSION TOMOGRAPHY/COMPUTERIZED TOMOGRAPHY (PET/CT)

PET, a non-invasive imaging modality, allows quantitative evaluation of the biological processes *in vivo* through emission of positrons from nuclear decay of an intravenously injected radiopharmaceutical. PET typically has higher sensitivity than conventional SPECT which allows the targeting of sites at much lower concentrations of radiotracers in the target area, allowing localization of small lesions.

One of the most commonly used radiotracers in MI is F-18 fluorodeoxyglucose (FDG), a compound approved by the FDA for clinical use. Since membrane glucose transporter (GLUTs) expression increases significantly in rapidly dividing cancer cells, FDG enters these cells producing preferential uptake and phosphorylation of FDG and permitting clinically meaningful imaging of the tumor. Besides FDG, there have been many advances in developing new class of radiotracers to diagnose cancer and to interrogate certain neurological processes. O-15 water (blood flow),[2] F-18 labeled fatty acids (fatty acid/thiokinase metabolism),[3] F-18 FLT (thymidine kinase),[4] F-18 fluoroestradiol (estrogen receptor targeting),[5] C-11 and F-18 choline (choline kinase/oncological imaging),[6-11] F-18 fluoromisonidazole (F-MISO; hypoxia imaging),[12,13] radiolabeled annexin (apoptosis),[14,15] F-18 FMDHT (androgen receptor imaging),[16,17] and F-18 FHBG and analogues (reporter gene targeting/imaging),[18-20] are some of the examples illustrating a wide scope and contribution of this imaging modality to molecular imaging field. Similarly, SPECT uses radionuclides which decay through a single photon emission branch. As with PET, SPECT also requires the use of radioactive probe for MI, albeit the chemistry involved in preparing those radioactive molecules could be quite different.

While the choice of radiotracer varies with application, several of these targeting probes are multifunctional and have a widespread utility across many diseases. Most of these probes are small molecules, have rapid clearance from blood pool (circulation), rapid washout from normal tissues and low metabolic disintegration. Our own and other researchers experience with C-11 choline in patients with prostate cancer has been quite impressive.[7,21-23] Patients with recently diagnosed prostate cancer were scanned using C-11 choline to localize the tumor. At the conclusion of the treatment regimen, the patients returned for a second C-11 choline scan to assess treatment efficacy. As shown in figure 1, PET/CT imaging with C-11 choline has been quite unequivocal in monitoring therapy outcome.[6]

Similarly, C-11 choline plays an impressive role in following treatment response in patients with esophageal cancer.[24] Several other PET imaging probes labeled with F-18 have also been developed over the years.[6,10,16,17,25,26,27] Whole body PET/CT imaging with 7α-[^{18}F] fluoro 17α-methyl 5α-dihydrotestosterone (F-18 FMDHT) in normal healthy volunteers is shown in figure 2. The radiotracer clears from most normal tissues following the hepatobiliary clearance. The initial uptake in the urinary bladder was low but increased after 60 min post injection, indicating its potential to clearly discern primary as well as metastatic prostate cancer from normal tissues.

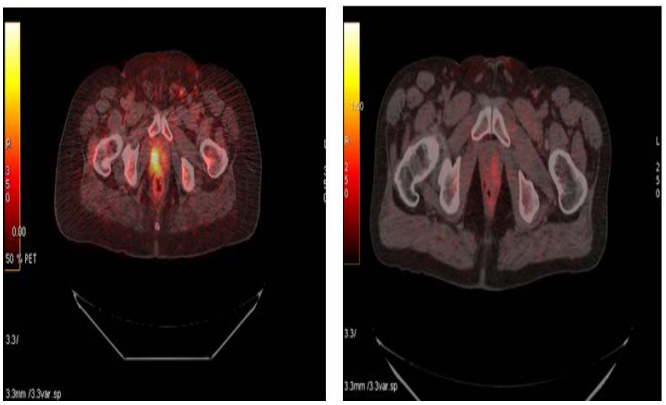

Figure 1: PET/CT image of a patient with prostate cancer before and after therapy. The left panel shows C-11 choline uptake in the prostate bed (scan acquired prior to therapy). After this scan patient underwent therapy. The right panel shows the PET/CT scan after patient completed therapy. No uptake of C-11 choline is noted in that region supporting the efficacy of C-11 choline to monitor prostate cancer therapy.

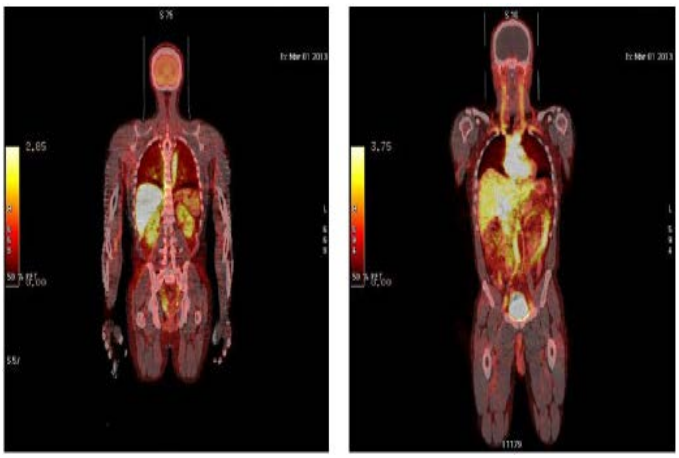

Figure 2: Distribution of F-18 FMDHT in normal healthy volunteer. Early image (left panel) show uptake of F-18 in the liver, spleen, kidneys. Late image of same patient (2 h post injection) show significant clearance of radioactivity from liver through the hepatobiliary path with radioactivity accumulation in the bladder.

While small molecular weight compounds have already been proven effective for molecular imaging applications, the role of large proteins such as antibodies has been steadily gaining momentum. One of the key parameters is to non-invasively visualize target molecules in altered cells by virtue of the target probe interaction at the molecular level. While some evidence suggests the clinical utility of Ab in molecular imaging, because of their large molecular size (150 kDa), the intact immunoglobulins remain in circulation[28] and may take a long time to accumulate in tumors (1-2 days) This slow uptake

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decreases the clinical utility of such a probe and investigators have been less than enthusiastic to consider such molecules as relevant for molecular imaging. Advancement in antibody engineering leading to small molecular weight constructs such as monovalent fragments (variable fragments Fv, single chain variable fragments (scFv), bivalent or bispecific diabodies, tribodies, Fab fragments and minibodies) raised the enthusiasm in the MI community to explore their utility in imaging. In comparison to the intact antibody, these smaller fragments, particularly the single chain antibodies $(scFv)_2$ show faster clearance from the blood and rapid peak tumor localization. Over the years, a number of antibody based tumor biomarkers have been developed for the diagnosis or treatment follow-up of specific cancers.[29-31] However, challenges to the effective exploitation of differentially expressed markers for the timely visualization of growing tumors remain. With the recent initial successes in preclinical and translational studies, several such antibody based entities are now FDA approved for clinic use. Some of the examples of antibody based radiopharmaceuticals are OncoScint CR/OV (Satumomab Pendetide) which targets the cell surface mucin-like glycoprotein antigen TAG-72 in colorectal and ovarian carcinomas, ^{111}In-labeled Oncoscint for pre-operative evaluation, monitoring recurrence,and detection of extrahepatic metastasis,[32] and ProstaScint (CapromabPendetide) for the diagnosis of metastatic prostate cancer.[33,34] A radionuclide with a longer half-life such as ^{124}I (4.18 d) has helped to further explore the use of whole IgG because the long half-life radionuclide allows for extended time for uptake in targeted sites while allowing for the clearance from normal tissues and from non-specifically circulating fraction[35] and maintaining enough radioactivity to produce a useful imaging signal. More recently, Zirconium-89 (^{89}Zr), a transition metal, has turned into an attractive choice of radionuclide for PET imaging due to its longer half-life (3.3 days) which is compatible with the pharmacokinetics of large molecules such as antibodies and their fragments. ^{89}Zr-labeled mAb has been proven to be stable in vivo and has provided better spatial resolution than that with ^{18}F FDG PET. Use of ^{89}Zr labeled mAbs as a scouting procedure preceding radioimmunotherapy has been proposed.[36-38] In one of the earliest studies, ^{89}Zr was labeled with mAb U36 recognizing the v6 domain of CD44, a tumor antigen overexpressed in head and neck tumors. This radioimmunoconjugate was used in preclinical models to confirm tumor targeting and estimate the dose deposition to tumor and normal tissues prior to radioimmunotherapy with ^{90}Y-labeled mAbs.[39]

Another radionuclide with attractive PET properties and compatible with large as well small molecular weight targeting molecules is copper-64 (^{64}Cu). Initial efforts using copper-64 explored the use of macrocyclic chelating agent 1,4,7,10-tetraazacyclododecane-N,N',N'', N'''-tetraacetic acid (DOTA). This radiolabeled small molecular weight antibody (minibody; 80 kDa) cleared rapidly from circulation and exhibited moderate tumor uptake shown by serial imaging of antigen positive tumors using preclinical models and micro PET imaging.

OPTICAL IMAGING

The use of optical imaging is also rapidly being adopted by molecular imaging scientists. Optical imaging includes fluorescence and bioluminescence based imaging probes. Optical imaging modalities are very promising due to their high sensitivity and specificity, low cost, portability of imaging instruments and absence of ionizing radiation. Further with the imaging is showing potential for clinical relevance. In fluorescence imaging, cells are labeled with dyes or proteins which emit light of a limited spectrum when excited. Bioluminescence methods use an enzymatic reaction between a luciferase enzyme and its substrate, luciferin, to produce photons which are converted to electrons and detected by a cooled Charged Couple Detector (CCD). This ultimately results in the detection of visible light through near-infrared light signals. Fluorescent molecules like Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) are used to observe the localization of proteins within a cell, to label specific proteins, or to monitor the production of a gene product.[40] For bioluminescence imaging, the bioluminescence reporter Firefly Luciferase (FL) and Renilla Luciferase (RL) are used frequently.

More recently, advances have been made in the field of infrared imaging. Near-Infrared imaging (NIR) has deeper penetration ability and higher sensitivity than the previously discussed optical imaging options. Ogawa et al., analyzed the efficiency of NIR flourophorindocyanine green (ICG) for *in vivo* imaging and produced encouraging results.[41]

ULTRASOUND IMAGING (US)

Ultrasound (US) is a cross-sectional imaging modality which uses sound-waves to produce interpretable images. Pulses of sound-waves of appropriate frequencies (usually 1-20 MHz depending on desired imaging depth) are emitted through arrays of transducers (probes).The sound-waves are reflected at tissue boundaries and these returning echoes are captured and reconstructed to produce a two-dimensional image of the plane being scanned. The resulting image is displayed using a grey-scale display corresponding to the intensity of the returning echoes. Ultrasound contrast agents are a relatively new concept and are designed to alter the absorption, reflection or refraction of the sound-waves which enhance the differentiation of the signal from the tissue containing the contrast agent from that of the surrounding sample. Targeted ultrasound contrast agents are site-directed contrast agents which specifically enhance the signal from pathologic tissue which would otherwise be difficult to distinguish from surrounding normal tissue. Microbubbles (MB) are US contrast agents which are gas filled structures encapsulated by lipid shell, polymer or proteins and when injected into the blood stream enhance signals from the target tissues.[42,43] Their low cost, easy transportability and utility to provide a discriminated signal from

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background with high sensitivity has made the MB contrast agent of choice for US imaging. Future research could allow specific ligands to be attached to the MB's surface which could lead to the accumulation of these agents in the target sites. Because of their large size, MB rarely extravagates from vascular tissue. Also, the MB targeted to the platelet glycoprotein IIb/IIIa integrin[44] and to fibrin[45] have been used for thrombus imaging. Fortunately, targeted MB based contrast imaging is even making functional characterization of tumor vasculature possible.

Although, there are manuscripts reporting that only a small number of MBs are required to produce adequate contrast for imaging purposes, further research is needed to assess the overall sensitivity of targeted microbbubles, determine the amount of antibody sufficient to get the target microbubble to the desired anatomic location, and to determine the comparative efficacy of MBs in comparison to nuclear and other optical imaging modalities.

MAGNETIC RESONANCE IMAGING (MRI) AND MAGNETIC RESONANCE SPECTROSCOPY (MRS)

MRI primarily uses the magnetic resonance signal produced from the hydrogen atoms in a sample under the influence of strong magnetic field for constructing three dimensional image sets. Positive and negative contrast agents containing metal ions are used to enhance MRI sensitivity. Positive contrast agents (appearing bright on MRI) are small molecular weight compounds usually containing gadolinium (Gd), manganese (Mn) or iron (Fe) as their active element. Negative contrast agents (appearing predominantly dark on MRI) are small particulate aggregates such as Super Paramagnetic Iron Oxide (SPIO) or Ultra-small Super Paramagnetic Iron Oxide (USPIO) particles which have an iron oxide core containing iron atoms covered by a polymer shell usually made of dextran or polyethyleneglycol. Although contrast enhanced MRI is a powerful imaging modality with sub-micrometer resolution (10-100 µm) and exquisite soft tissue contrast, its major drawbacks are low sensitivity, low retention of positive contrast agents, relatively long imaging times, and the necessity to inject large amounts of contrast to obtain quality images.

Since conventionally used contrast agents like Gd lack specificity, targeted contrast agents are being developed by conjugating paramagnetic compounds to various peptides, ligands, antibodies and antibody fragments that target specific tumor cell surface antigens. Superparamagnetic Iron Oxide (SPIO) is another class of negative contrast agents used in MR imaging.

Nanoparticles are nano-sized carriers used for delivery of various pharmaceuticals. In recent years, there has been intense effort to develop multifunctional nano-carriers for the targeted delivery of diagnostic and therapeutic agents. These multifunctional nano-carriers could incorporate antibodies for specific and effective tumor targeting and delivery of therapeutic payloads. For example, nano-shells are optically tunable nanoparticles that contain a dielectric core surrounded by a thin gold shell. These nano-shells may be designed to scatter and/or absorb light over a broad spectral range. Thus depending on wavelength of light chosen, these nanoparticles can be utilized for imaging and for photo thermal therapy.

OTHER IMAGING MODALITIES

Raman spectroscopy is based on the inelastic scattering of light during its interaction with matter. Although the light scattered from biological samples is usually weak, the intensity of scattered light increases tremendously in the vicinity of metallic nanoparticles. These metallic nanoparticles, on excitation at a particular wavelength, exhibit surface plasmon resonance and resonate light. This resonation results in enhanced scattering from these metallic particles which result in increased contrast when compared to the surrounding molecules. The increased contrast is basis for Surface Enhanced Raman Spectroscopy (SERS). Due to its ability to detect picomolar amounts of the targeted molecule, SERS is becoming a predominant imaging modality. SERS provide multiple advantages over existing imaging modalities including the unique spectral properties of SERS nanoparticles with easily resolved narrow peak which allows for multiplex imaging ,lower toxicity due to inert nature of SERS nanoparticles (Gold, silica). Moreover, colloidal gold particles coated with a protective layer of polyethylene glycol (PEG) exhibit excellent *in vivo* biodistribution and pharmacokinetic properties upon systemic injection, and SERS probes provide enhancement of the Raman scattering of adjoining molecules by as much as 10^{14}-10^{15} which can lead to the detection of a single molecule.[46,47] SERS is becoming the modality of choice when localized and surface tumors. However, its use remains limited in imaging deep tumors due to shallow depth of penetration associated with Raman microscopes and the non-specific uptake of these particles.[48]

CONCLUSION

A wide array of imaging modalities is available to the preclinical research community and many of these modalities have been adopted by the clinic. Nonetheless, the efforts to further optimize probes to more precisely target cellular level processes with high specificity and selectivity continues to be an area of much research effort. As we gain more knowledge on the molecular interactions of various disease states, new and novel probes are being considered, explored, and designed. Along with probe development, or perhaps because of it, there has been great progress in enhancing the capabilities of imaging instruments and techniques to further help visualize these molecular processes with increased sensitivity and resolution. As the molecular imaging field is evolving and scientists and physicians are recognizing its importance, industries are tak-

-ing note of the strength of this modality. Several pharmaceutical companies have either entered in collaboration with researchers from various academic institutions or have developed an in-house molecular imaging program. It is only a matter of time when molecular imaging will become an integral part of drug-development to provide routine standard of care in the clinic.

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Mini Review

Corresponding author

Satoru Matsuda, MD

Department of Food Science and Nutrition, Nara Women's University Kita-Uoya Nishimachi, Nara 630-8506, Japan

Tel. +81 742 20 3451

E-mail: smatsuda@cc.nara-wu.ac.jp

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Regulation in Cell Cycle *via* p53 and PTEN Tumor Suppressors

Yasuko Kitagishi*, Satoru Matsuda*, Akari Minami, Yuna Ono, Atsuko Nakanishi and Yasunori Ogura

Department of Food Science and Nutrition, Nara Women's University, Kita-Uoya Nishimachi, Nara 630-8506, Japan

ABSTRACT

One of the target effectors of p53 transcription factor is the Phosphatase and Tensin homologue deleted on chromosome 10 (PTEN) which has protein phosphatase activity and lipid phosphatase activity that antagonizes PI3K activity. Cells that lack PTEN have constitutively higher levels of PIP3 and activated downstream targets. Both p53 and PTEN are tumor suppressors that act by inhibiting cell cycle progression and promoting apoptosis. Germline mutations in p53 and PTEN cause Li-Fraumeni syndrome and Cowden syndrome, respectively. The p53 cooperates with PTEN, which might be an essential blockage in development of cancers. PTEN protects p53 from MDM2-mediated degradation, whereas p53 can enhance the transcription of PTEN. This review summarizes the function of PTEN and p53 in cell cycle regulation. We will also discuss the role of PTEN signaling through its interaction with p53 and MDM2 pathways for the potential implications in the cell cycle regulation.

KEYWORDS: p53; PTEN; AKT; MDM2; Protein interaction; Protein degradation; Cell signaling; Cell cycle regulation.

ABBREVIATIONS: HDM2: Homologue of MDM2; MDM2: Murine Double Minute 2; NEDL1: NEDD4-like ubiquitin protein Ligase-1; PDZ: PSD-95, DLG1, and ZO-1; PEST: Proline, glutamic acid, Serine and Threonine; PTEN: Phosphatase and Tensin homologue deleted on chromosome 10; PIP3: Phosphatidylinositol 3,4,5-triphosphate ; PIP2: Phosphatidylinositol 4,5- bisphosphate; PI3K: Phosphoinositide-3 Kinase; PTP: Potein Tyrosine Phosphatase; RTK: Receptor Tyrosine Kinase; ROS: Reactive Oxidative Species.

INTRODUCTION

Mechanisms of cell arrest in cell cycle are predominantly governed by p53 tumor suppressor[1] that is a transcription factor. The p53 protein is able to induce G1 arrest of the cell cycle by trans-activating several downstream molecules. Germinal mutations of the p53 gene constitute an etiological genetic base of Li-Fraumeni syndrome, which is a rare hetergoeneous autosomal dominant inherited cancerous disorder.[2] The p53 is at a midpoint of cellular signaling networks that are activated by stress signals including DNA damages.[3] PTEN (Phosphatase and Tensin homolog deleted in chromosome 10) is also a tumor suppressor gene that is deleted or mutated in a variety of human cancers.[4,5] Germ line mutations in PTEN are the cause of PTEN hamartoma syndromes (Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, PTEN-related Proteus syndrome, Proteus-like syndrome) with increased risk for a development of cancers.[6] Characterization of PTEN protein has showed that it is a phosphatase and can modulate signal-transduction pathways that involve lipid second messengers.[7] PTEN prevents an activation of PI3K/AKT pathway by dephosphorylating the membrane phospholipid PIP3, and thus influence cell survival signaling.[7] Loss of PTEN results in increased AKT recruitment to the plasma membrane, and activates the signaling pathway.

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The PTEN has been shown to be involved in a complex network on interactions with p53 (Figure 1). Although they are functionally distinct, reciprocal cooperation has been proposed, as PTEN is thought to regulate p53 stability, and p53 to enhance PTEN transcription. Once PTEN is lost, however, the p53 pathway is strongly activated.[8,9] Furthermore, an absence of PTEN cooperates with an absence of p53 to promote cancer,[10] supporting a model for cooperative tumor suppression in which p53 is an essential failsafe protein of PTEN-deficient tumors.[8] Inactivation of tumor suppression may be caused by lack of these key interaction partners. Recent studies have revealed a functional ubiquitin ligase for tumor suppressors play a pivotal role in tumor cell survival.[11,12] They may regulate the stability of key tumor suppressors. Mutations found in genes such as p53 and PTEN have emerged as high penetrance susceptibility genes and are clinically relevant for determination of cancer risk. In addition, there are multiple nodes of crosstalk between PI3K/AKT/PTEN and p53 signaling pathways.[13] In this review, we summarize the current research and our view of how and when PTEN and p53 with their partners to transduce signals downstream and what are the implications for cell cycle-associated biology in cancer.

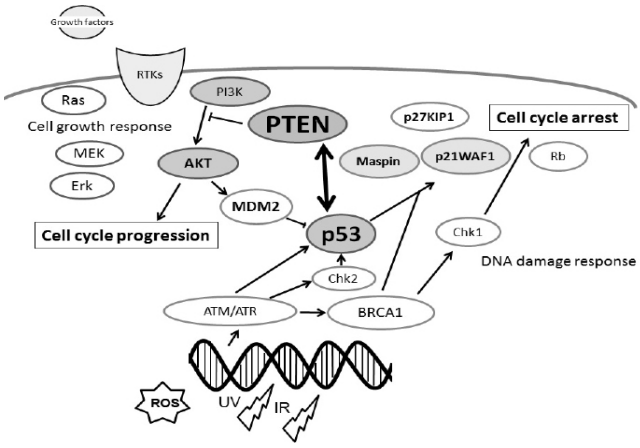

Figure 1: Schematic representation of the integrative model of tumor suppressors signaling including PTEN and p53. Typical examples of molecules known to act on the DNA damage response and cell cycle progression *via* the regulatory pathway are shown. Note that some critical pathways have been omitted for clarity.

FUNCTIONAL CHARACTERISTICS OF PTEN AND P53

The human genomic PTEN locus consists of 9 exons on chromosome 10q23.3 encoding a 5.5 kb mRNA that specifies a 403 amino-acid open reading frame.[14,15] The translation product is a 53 k Da protein with homology to tensin and protein tyrosine phosphatases. PTEN activity can be regulated by posttranslational regulation including phosphorylation, acetylation, and oxidation.[16] Methylation of the PTEN promoter region can result in transcriptional silencing of the PTEN gene.[17] Schematic structure of the predicted PTEN protein is shown in Figure 2. PTEN negatively regulates the activity of PI3K/AKT signaling through converting Phosphatidylinositol 3,4,5-triphosphate (PIP3) into Phosphatidylinositol 4,5- bisphosphate (PIP2). The PIP3 is the principal second messenger of the PI3K pathway that mediates Receptor Tyrosine Kinase (RTK) signaling to the survival kinase AKT. Activated AKT transfers a phosphate group to target proteins involved in cell survival, cell cycling, proliferation, and cell migration, which are all critical for tumor development.[18,19] Generally, AKT is activated by growth factors and the RTK that activates PI3K. Upon activation, PI3K phosphorylates the inositol ring, which in turn serves to anchor AKT to the plasma membrane, where it is phosphorylated and fully activated by the 3-phosphoinositide-dependent kinases PDK1 and PDK2. PTEN acts as regulator of maintaining basal levels of PIP3 below a threshold for those signaling activation. PTEN protein consists of N-terminal phosphatase, and C-terminal C2, and PDZ (PSD-95, DLG1, and ZO-1) binding domains. The PTEN CX5R(S/T) motif resides within an active site that surrounds the catalytic signature with three basic residues, which are critical for PTEN lipid phosphatase activity. The structure provides PTEN with its preference for acidic phospholipid substrates such as PIP3. Overexpression of the PTEN induces growth suppression by promoting cell cycle arrest, which requires lipid phosphatase activity.[20,21] Overexpression of PTEN also correlates with decreased levels and nuclear localization of cyclin D1[22], a cell cycle key molecule regulated by AKT. One mechanism by which PTEN induces cell cycle arrest is by regulating AKT activity so that levels of the cell cycle inhibitor p27kip1 are increased.[23] However, despite the main role of PTEN as a negative regulator of the PI3K pathway, studies report many tumor suppressive activities for PTEN that are exerted from within the nucleus, where catalysis of PIP3 does not seem to represent a main function of this enzyme.[24] The nuclear PTEN activities may include the regulation of genomic stability and gene expression.

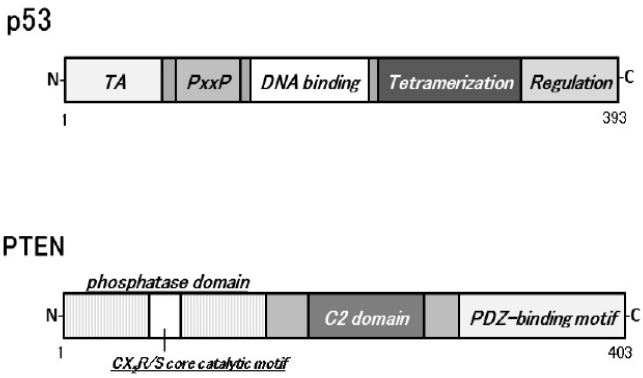

Figure 2: Schematic structures of p53 and PTEN proteins. The predicted consensual domain structures for each protein are depicted. The functionally important sites are shown. TA= transactivation domain; PxxP= proline rich region; C2 domain= a protein structural domain involved in targeting proteins to cell membranes; PDZ= a common structural domain in signaling proteins (PSD95, Dlg, ZO-1, etc)

The p53 gene, located on chromosome 17p 13.1, encodes a nuclear 393 amino-acids protein which is a transcriptional factor regulating cell cycle and apoptosis when DNA damage occurs (Figure 2). The p53 tumor suppressor plays a pivotal role in regulating cellular processes including cell cycle arrest, apoptosis, cell metabolism and cell senescence. Inactivation of p53 gene is a common event in the development of most types of cancers[25], suggesting that p53 plays a critical role in preventing normal cells from becoming malignant

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cells. The p53 germline mutations may occur in up to 1: 5000 individuals.[26] The importance of p53 as an inherited cancer susceptibility gene has also been demonstrated in Li-Fraumeni syndrome.[26] Multiple mechanisms have been revealed to collectively achieve the regulation of p53 activity,[27,28] which determines the selectivity of p53 for specific transcriptional targets, resulting in precise control of the p53 activity. Release of p53 from repression by factors such as Mdm2 and MdmX may be a key step in the physiological activation of p53.[28] Activation of p53 function involves its increased DNA-binding ability, transcriptional activation, increased expression of p53 target genes, associated with cell cycle progression and apoptosis.

FUNCTIONAL INTERPLAY AMONG P53, PTEN, AKT AND MDM2

An important p53 function is to act as a transcription factor by binding to the specific DNA consensus sequence in responsive genes. The PTEN and p53 complex enhances p53 DNA binding and transcriptional activity,[29] which may increase the synthesis of PTEN and p21waf1 that is an important protein involved in cell cycle arrest.[30] One way by which p53 inhibits production of PIP3 indirectly is by inducing the expression of this PTEN.[31] Under hypoxic conditions, PTEN and p53 form a complex in the nucleus and induce expression of additional tumor suppressor Maspin.[32] In other words, the nuclear PTEN and p53 coordinates the induction of maspin. Loss of PTEN attenuates the induction of Maspin even in the presence of wild type p53. Integration of PTEN and p53 into a common pathway for Maspin-induction may constitute a strong tumor suppressors network.[33] The ability of p53 to induce cell cycle arrest or apoptosis can be antagonized by survival signals. The PI3K dependent activation of AKT indirectly leads to the inhibition of p53 functions by activating another tumor suppressor MDM2.[34] Several studies have implicated AKT in modulating DNA damage responses and genome stability.[35] In addition, activation of AKT has the potential of reducing the p53-mediated cell cycle checkpoints through phosphorylation and sequestration of p21waf1, and *via* the enhanced degradation of p53.[36] PTEN also plays a critical role in DNA damage repair and DNA damage response through its interaction with p53 pathways in an AKT-independent manner.[37] Nuclear PTEN is sufficient to reduce tumor progression in a p53 dependent manner. It has also been suggested that nuclear PTEN play a unique role to protect cells upon oxidative damage and to regulate carcinogenesis.[38] Studies suggest that nuclear PTEN mediates DNA damage repair through modulating the activity of DNA repair molecules.

MDM2 is an oncoprotein that controls carcinogenesis, whose mRNA level is transcriptionally regulated by the p53 in response to DNA damage such as oxidative stress.[39] In addition, the MDM2 protein and subcellular localization are post-translationally modulated by AKT.[40] Besides PTEN inhibits the PI3K/AKT signaling, PTEN promotes translocation of MDM2 into the nucleus. In addition, PTEN modulates MDM2 transcription and isoform selection by negatively regulating its promoter.[41] In PTEN-null cells, MDM2 promoter activity is up-regulated, resulting in increased MDM2 expression. Furthermore, PTEN controls MDM2 promoter activity through its lipid phosphatase activity, independent of the p53 activity.[29] Although another transcription factors may be able to modulate MDM2 transcription, they have been characterized to work through the p53 responsible promoter.[42] MDM2 also regulates the activity of p53 protein by exporting of nuclear p53 protein into the cytoplasm and by promoting the degradation of the p53 protein. PTEN up-regulates the p53 level as well as its activity by down-regulating MDM2 transcription and p53 binding activity.[43] However, in the absence of p53, PTEN may have a role inhibiting MDM2-mediated carcinogenesis through regulation of MDM2 transcription. The ability of PTEN to inhibit the nuclear entry of MDM2 increases the cellular content and transactivation of p53 to promote the induction of genes such as p21waf1.[44]

Consequently, p53 and AKT influence the process of apoptosis in opposite ways. The AKT promotes cell survival by suppressing pro-apoptotic proteins such as Bad through the phosphorylation.[45] There are cross talks between p53 and AKT involving gene transcription as well as posttranslational protein modifications. One way by which p53 inhibits indirectly PIP3 production is by repressing the catalytic subunit of PI3K. Subsequent p53-induced expression of PTEN causes the p53-PTEN interaction, which also suppresses the cell survival machinery of AKT pathway. AKT kinase phosphorylates MDM2 to translocate into the nucleus.[46] In addition, PTEN associates with p53 and regulates the transcriptional activity of p53 by modulating its DNA binding.[29] PTEN is required for the maintenance of p53 acetylation, which is required for target gene transcription.[47] One side of the PTEN function as a tumor suppressor is achieved through this stabilization of the p53 protein. PTEN has been shown to interact with p53 and prevent its degradation by excluding a portion of p53 protein from the p53 and MDM2 complex. AKT mediates MDM2 nuclear translocation by its phosphorylation. MDM2 negatively regulates p53 by binding for destabilization in the nucleus.[48] Attenuation of the AKT pathway by PTEN protects p53 from MDM2 mediated degradation and inactivation. The p53 and MDM2 complex transports from the nucleus into the cytoplasm where MDM2 serves as an E3 ubiquitin ligase.[49] Therefore, p53 and MDM2 form a regulatory feedback loop in which p53 positively regulates MDM2 expression, whereas MDM2 negatively regulates the level of p53 protein. Inactivation of either p53 gene or PTEN gene results in lower protein levels of the other gene.

The instability of PTEN correlated with its missense mutations has been shown to involve protein interactions. PTEN may be regulated by ubiquitin-mediated proteasomal degradation, a common mechanism to control protein levels. In cells, ubiquitin ligase NEDD4-1 negatively regulates PTEN stability by catalyzing PTEN ubiquitination.[50] Because deletion of the C2 domain of PTEN makes the protein unstable and

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accelerates the protein degradation, the C2 domain of PTEN seems to regulate itself through maintaining the protein stability.[51] In addition, the C-terminus of PTEN contains two PEST (proline, glutamic acid, serine and threonine) sequences involved in ubiquitin protein degradation pathway. Treatment of cells with proteasome inhibitors can cause an increase of PTEN protein level.[52,53] So, several NEDD4-like E3 similarly regulate p53. Multiple NEDD4-like E3 show ligase independent function and most of NEDD4-like E3 are commonly regulated by phosphorylation, ubiquitination, translocation, and transcription in cancer cells. Functional interaction of NEDD4-like ubiquitin protein Ligase-1 (NEDL1) with p53 might contribute to the induction of apoptosis in cancerous cells.[54,55] Casein kinase II-mediated phosphorylation stabilizes the PTEN protein by preventing the proteasomal degradation, which results in increased PTEN activity and a corresponding reduction in AKT activation.[56] Interestingly, inhibitors of Casein kinase II also activate p53 function in wild-type but not in p53 mutant cells, which increases senescence in the p53-dependent manner.[57] It seems that Casein kinase II may control the PTEN and the p53 in balance.

INVOLVEMENT OF PTEN-P53-AKT-MDM2 LOOP IN CELL CYCLE REGULATION

It has been proposed that low levels of p53 induce cell cycle arrest, whereas high levels of p53 induce apoptosis.[58] Probably, p53 can bind to pro-arrest genes of cell cycle with high affinities but associates with pro-apoptotic genes with low affinities.[59] The levels of p53 could vary and is positively related to the amount of DNA damage.[60] Activation of AKT, on the other hand, can overcome both the p53-independent G2/M cell cycle checkpoint and apoptosis induced by the DNA damage. In addition, growth factor-activated AKT signaling supports progression of cell cycle by acting on several factors involved in the G1/S or G2/M transitions. Because the ability of p53 to induce cell cycle arrest or apoptosis can be antagonized by survival signals, which indirectly leads to the inhibition of p53 functions by activating its negative regulators.[34] Given the ability of PTEN to stabilize p53 protein through antagonizing the AKT-MDM2 pathway[29,44] or by directly increasing p53 acetylation,[47] decreased p53 activity in PTEN-deficient tumor cells could be expected. Stabilization and constant levels of the p53 may trigger apoptosis in damaged cells. The target genes of p53 are selectively induced to control cell fate. Consequently, the cell fate may be determined by the levels of p53.[61,62] Constitutive activation of AKT in PTEN-deficient cells should down-regulate p53 transcriptional activity and block p53-induced p21waf1 induction.[44] The PTEN-p53-MDM2-AKT loop in cell cycle regulation now becomes dominant (Figure 3). In addition, PTEN and p53 is known to interact and regulate each other at the transcription as well as protein level, which could be at the important control machinery for switching between survival and death. These cross talks are frequently a combination of reciprocally antagonistic pathways, which may often serve as an added regulatory effect on the expression of key genes involved in cancer. Interestingly, soy isoflavone genistein induces an auto-regulatory loop between PTEN and p53 to promote cell cycle arrest.[63] The induction of PTEN expression and nuclear accumulation by genistein elicits a sequence of PTEN-dependent increased nuclear p53 accumulation, enhanced PTEN/p53 physical int eraction, increased recruitment of the PTEN/p53 complex to

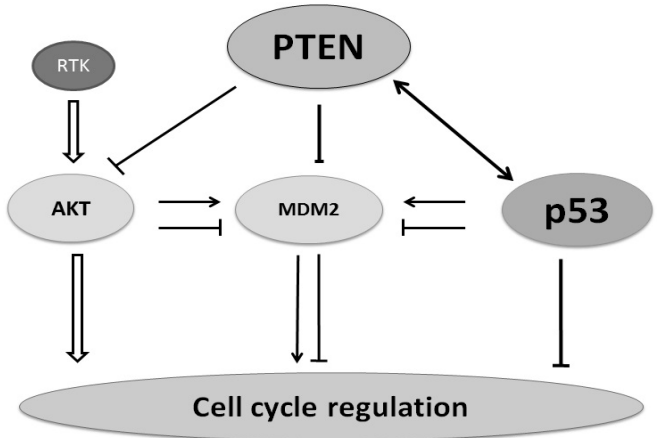

Figure 3: Suggestion of various regulatory loops involving the PTEN-p53-AKT-MDM2 network on cell cycle progression. Interactions are shown as arrows to mean activation, while hammerheads to mean inhibition. Note that some critical pathways have been omitted for clarity.

the p53 binding sites of the PTEN promoter, attenuated expression of proliferative genes cyclin D1and pleiotrophin gene expression, and promotion of cell cycle arrest.[63] Genetic variants in the PTEN, p53, AKT, and MDM2 tumor suppressor oncoprotein network may play roles in mediating the susceptibility to cancer.[64] It has been shown that zinc deficiency modulates the PTEN-p53-MDM2-AKT signaling axis in normal prostate cells.[65]

PERSPECTIVE

The tumor suppressor p53 predominantly induces cell cycle arrest or apoptosis in the DNA damage response. In regular unstressed cells, p53 may be kept at low levels by its negative regulator MDM2. This positive feedback loop among PTEN -p53- AKT-MDM2 may function for the precise regulation of the cell cycle (Figure 3). The numerous interactions may support the biological plausibility that the combination of variants of the PTEN -p53- AKT-MDM2 network could result in more comprehensive and accurate estimates of risk than can be obtained from a single variant. Accordingly, germline genetic testing for mutations in these genes allows for the identification of individuals at increased risk for cancers, which are the state of running off from the cell cycle regulation. However, they may be regulated and interact each other at multiple levels including transcription, protein modulation, and protein stability. For example, increased nuclear localization of PTEN may promote nuclear retention of p53 and the subsequent transactivation by the PTEN and p53 complex of the PTEN promoter. By the way, what are the substrates of PTEN in that situation? Understanding the regulation is crucial for the effective

design of novel cancer therapeutics. In addition, it is important to investigate the functional linkage between PTEN, p53 and MDM2 isoforms in human cancer samples, and elucidation of interaction-specific functions may provide insight into regulatory aspects of these tumor suppressors. Further mechanistic studies are needed in order to understand the more precise molecular mechanisms.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Review

*Corresponding author
Sean L. Kitson, PhD
Department of Biocatalysis and Isotope Chemistry, Almac, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, UK
E-mail: sean.kitson@almacgroup.com

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Application of Radionuclides and Antibody -Drug Conjugates to Target Cancer

Sean L. Kitson*

Department of Biocatalysis and Isotope Chemistry, Almac, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, UK

ABSTRACT

Radionuclide therapy and antibody-drug conjugates are used to locate and kill cancer cells by the utilisation of monoclonal antibodies. These bio-vectors are able to transport a cytotoxic drug payload and/or radiation in the form of alpha or beta particles to bind onto antigen specific cancer cells initiating apoptosis. This inaugural article aims to deliver a brief account of these targeted therapies in the treatment of oncological disease states such as leukaemia, non-Hodgkin's lymphoma, neuroendocrine tumours, breast cancer and prostate cancer bone metastases.

KEYWORDS: Targeted alpha therapy; Radionuclide therapy; Antibody-drug conjugates; Monoclonal antibody, Alpharadin®; Xofigo®; Bexxar®; Zevalin®; Adcetris®; Kadcyla®.

INTRODUCTION

A century ago, the bacteriologist Paul Ehrlich (1908 Noble Prize) a pioneer of chemotherapy and haematology, first postulated the concept of targeted therapy towards the treatment of disease causing agents.[1] This concept was to create an ideal therapeutic agent termed the 'magic bullet' which went directly to specific cellular targets in order to attack the disease. Currently, Ehrlich's vision is now being realized in the treatment of cancer with the development of targeted therapies, mainly based on monoclonal antibodies.[2]

A major breakthrough was made in 1975, by the Nobel Prize winners Milstein and Köhler, in the development of hybridoma technology. This technology platform revolutionised the production of antibodies by having a single specificity towards the cognate antigen, in the development of targeted therapies.[3] Moreover, this approach is being exploited by several biopharmaceutical companies to develop strategies for delivering radionuclides to image and destroy a variety of cancers including adequate cytotoxic drug payloads.[4]

These cytotoxic drug payloads are utilised in the development of Antibody-Drug Conjugates (ADCs) and include the anti-neoplastic agents: Mono Methyl Auristatin E (MMAE) and mertansine (DM1) to target the microtubules in cancerous cells. These other payloads include the DNA damaging agents calicheamicins and duocarmycins extending to the topoisomerase II inhibitors doxorubicins and camptothecins.[5] All of these cytotoxic drugs have demonstrated *in vitro* potency against several tumour cell lines, down to the picomolar level. This compares to first generation ADCs using nanomolar amounts of doxorubicin.[6]

Currently, other emerging drug payloads such as the sequence selective DNA alkylating agents called Pyrrolo Benzo Diazepines (PBDs) will form the basis of the next generation of ADCs.[7] These PBDs have shown to be ten thousand times more potent than systemic chemotherapeutics and nearly a thousand times more potent than other cytotoxins used in ADCs.

The ideal treatment plan for a patient is first to locate the cancerous site(s) by using a radionuclide antibody capable of imaging the tumour volume. The following imaging modalities can be applied: planar imaging; Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). These techniques can be extended to state-of-

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the-art clinical hybrid imaging systems which combine SPECT (or PET) with Computed Tomography (CT) and more recently PET scanners with functional magnetic resonance imaging (f-MRI) instruments.[8,9]

However, if the cancer site is shown to retain an appropriate level of the antibody - through the application of these imaging techniques - based on gamma or positron emitters: it would be reasonable for the patient to receive a therapeutic dose of the same antibody - labelled with a radionuclide emitting alpha or beta radiation - which ever proves more capable of killing the cancer cells.

The limitation of utilising murine antibodies has been circumvented by the use of chimeric, humanized, or fully human monoclonal antibodies.[10] The challenge of targeting solid tumours with alpha and beta radiation is the dilemma of inducing a sufficient response on the cancerous mass without producing lethal toxic side effects. Therefore, it is paramount to analyse and control the radiation dose being delivered to tumour site(s) and compare the effects of this dose on the surrounding healthy cells.

Consequently, it is important to calculate the risk to other normal and/or non-neoplastic sites, capable of concentrating radioactivity, especially in the excretory organs (e.g. kidneys).[11] To help to define the risk, it is important to obtain pharmacokinetic data about the therapeutic radionuclide. This will enable a calculation of the percentage of injected dose per gram tissue therefore limiting normal tissue damage.[12-14]

The majority of precedents set by Radio-Immuno Therapy (RIT) were made by antibodies labelled with beta emitters (e.g. iodine-131). Today, after extensive research and clinical trials, RIT therapy against various cancers has now been accepted.[15] A continuation of research in the application of alpha emitters to treat cancer has been proposed for radiolabelling of many molecules, transported by various bio-vectors such as monoclonal antibodies.[16]

The main emphasis is the utilisation of radiolabelled antibodies as agents for Radio-Immuno Therapy (RIT). Following labelling with alpha emitters, Radionuclide Antibody-Conjugates (RACs) became the prototype for Targeted Alpha Therapy (TAT) using other targets and bullets, as in the case of peptides[17] and somatostatin receptors.[18]

Ongoing clinical trials have shown that somatostatin receptor peptides labelled with the beta emitters yttrium-90 and lutetium-77 have been effective in the treatment of neuroendocrine tumours.[19]

RADIONUCLIDES TARGETING CANCER

Currently, there are around 100 radionuclides that emit alpha radiation; the majority of them produced in nuclear reactors. Only a few are considered useful as therapeutics agents. These include bismuth-213 (generator produced),[20] astatine-211 (cyclotron produced),[21] actinium-225 (generator produced)[22] and thorium-227 (generator produced).[23] These radiolabelled therapeutic agents transported by bio-vectors such as monoclonal antibodies can be utilized in the treatment of a variety of cancers such as lymphomas, leukaemia and melanomas.[24] This is demonstrated further on by the ability of Xofigo® to form complexes within the area of bone metastases.[25] This is due to theactive moiety radium-223, in the form of radium-223 dichloride, to mimic calcium and the ability to complex with the bone mineral hydroxyapatite.[26]

A clinical precedent, on the practice of using alpha therapy towards bone metastases, with radium-223 dichloride (half-life=11.4 days) marketed as Alpharadin® became a first-in-class therapeutic.[27] In May 2013, Alpharadin® now called Xofigo®, was given FDA approval to treat patients with castration-resistant prostate cancer, symptomatic bone metastases with no known visceral metastatic disease.[28]

Xofigo® is the first and only alpha particle-emitting radioactive therapeutic agent approved by the FDA that has demonstrated improvement in overall survival rates.[29] To date, the most promising advance in cancer therapy is connected to the evolvement of using Radiolabelled Antibody-Conjugates (RACs) to deliver alpha particles.[30]

The basic principle of the TAT technique relies on the emission of alpha particles in which the radionuclide (e.g. actinium-225, bismuth-213, astatine-211) is held in a crown shaped chelate (e.g. derivatives of DTPA, DOTA), connected preferably to a low molecular weight drug (Figure 1). Alternatively, it may be more frequently linked to a monoclonal antibody, antibody fragments or peptide *via* a linker-chelate.[31,32] Therefore, it is paramount to get the right combination of radionuclide, linker-chelate and/or peptide, drug substrate or antibody for a particular cancer.[33-35] This is to ensure that an adequate amount of radiation is delivered to the cancer site by targeting the specific antigen (e.g. CD20) to annihilate it.

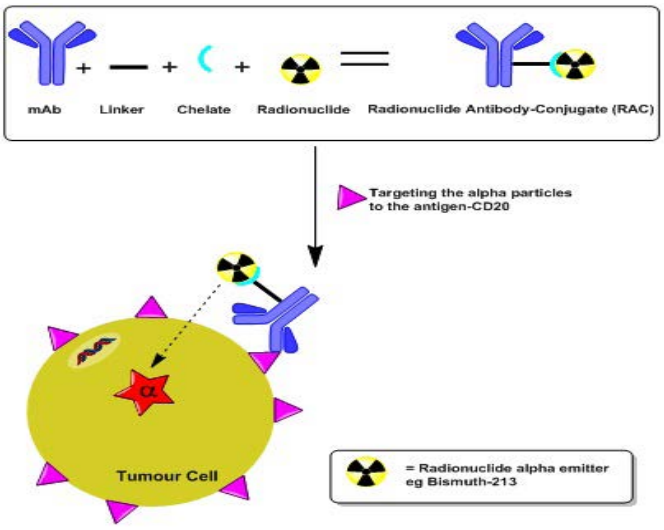

Figure 1: TAT targetingCD20 antigens on tumour cells

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Several TAT research groups have shown that the ideal radionuclide for this approach to be effective must have the following basic parameters.[36-38]:

- The radionuclide must emit an energy lower than 40 keV;
- Alpha particles have a short pathlength (50-80 microm) and high linear energy transfer of approximately 100 keV/mi crom;
- The radionuclide should have an ideal half-life of 30 min utes to 10 days to allow for logistics and treatment plan for the patient;
- For the generation of 'medical' radionuclides, the daugter radionuclide must be stable with a half-life greater than 60 days;
- The radiopharmaceutical in the form of kits and/or synthsis must be able to incorporate the radioactive label into carrier substrates as rapidly as possible for patient use.

Numerous clinical trials have utilised a wide range of bio-vectors to target cancer and include: HuM195 for acute myelogenous leukaemia;[39] Astatinated MX35-F(ab')2 monoclonal antibodies for ovarian cancer;[40] Radium-223 dichloride for bone metastases;[41] Murine 9.2.27 to target the Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP) antigen on melanoma;[42] CD20 antigen for lymphoma[43] and human IgG2/mouse chimeric anti-tenascin 81C6 forglioblastoma multiforme.[44]

Currently several preclinical trails include the bio-vectors: Monoclonal antibody C595 labelled with bismuth-213 to target MUC1 gene expressed by the prostate;[45] PA12 human recombinant protein to target urokinase-type Plasminogen Activator (uPA) system which is expressed in several types of cancer (e.g. breast cancer);[46] Monoclonal antibody J591 to target the Prostate Specific Membrane Antigen (PSMA)[47] and Bevacizumab (Avastin®) in the treatment of recurrent glioblastoma.[48]

Currently, other approaches to target cancer include the following FDA approved radiopharmaceuticals:

This targeted approach continues with Bexxar®, which contains the antibody tositumomab, radiolabelled with iodine-131 to target the CD20 antigen.[49] The patient first receives tositumomab, followed by the infusion of tositumomab radiolabelled with iodine-131. This is the same antibody covalently bound to the radionuclide iodine-131. The iodine-131 emits both beta and gamma radiation and decays with a half-life of 8 days (Figure 2). A successful clinical study involving 40 patients led to the approval in 2003 of Bexxar® for the treatment of rituximab-refractory, low-grade, follicular non-Hodgkin's lymphoma.[50]

The unique feature of Zevalin® is that it can be used to target the CD20 antigen on B-cell non-Hodgkin's lympho

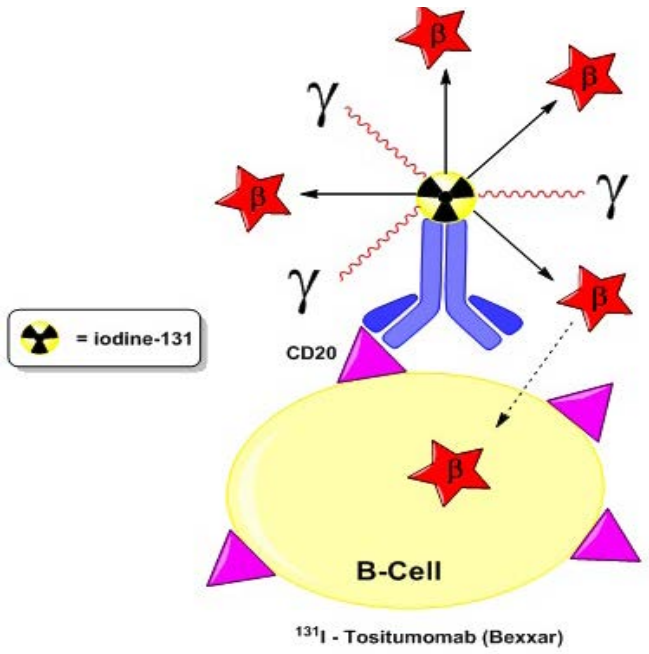

Figure 2: Bexxar® firing beta and gamma radiation to kill B-cells (Adaptation from Kitson et al. [51])

-ma to allow imaging and in therapy to destroy it. The radiopharmaceutical is first labelled with the radiometal indium-111, using tiuxetan chelation. This gamma-emitter is transported to the lymphoma sites by the monoclonal antibody ibritumomab which detects B-cells. SPECT imaging then can be used to verify that the antibody is properly distributed within the body.[52] The indium-111 is swapped with radionuclide yttrium-90, to transport beta particles to kill B-cells (Figure 3).

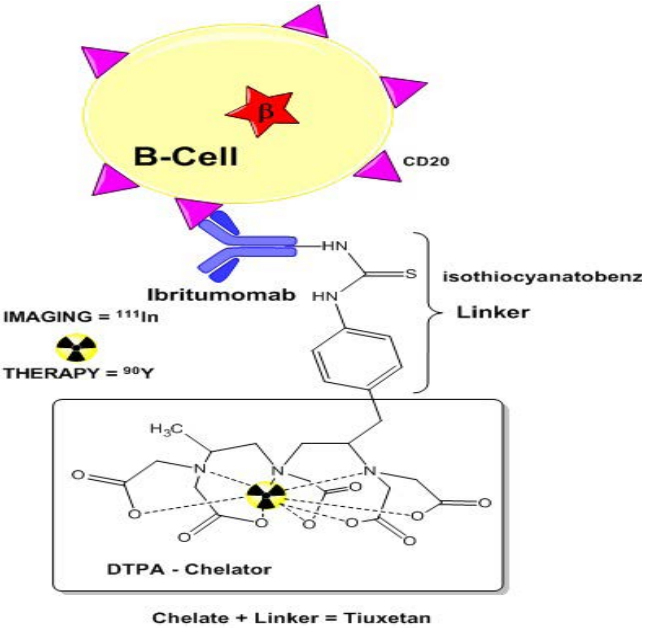

Figure 3: Zevalin® firing beta-particles at B-cells(Adaptation from Kitson et al. [51])

Zevalin® therapy has useful indications for relapsed or refractory, low grade or follicular, B-cell non-Hodgkin's

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lymphoma.[53] In 2002, the FDA gave approval for Zevalin® to be used in treatment of relapsed or refractory low-grade follicular or transformed B-cell non-Hodgkin's lymphoma; including patients with rituximab refractory follicular non-Hodgkin's lymphoma. In 2008, Zevalin® was approved as the first-line consideration for follicular lymphoma in the European Union.[54]

ANTIBODY-DRUG CONJUGATES (ADCs) TARGETING CANCER

These immunotherapeutic agents called Antibody Drug Conjugates (ADCs), target specific antigens particularly on cancer B-cellssuch as CD19, CD20, CD21, CD22, CD40, CD72, CD79b and CD180.[55] In 2011, the FDA gave approval to Adcetris® to treat Hodgkin's lymphoma and systematic anaplastic large-cell lymphoma.[56] The continued success of this therapeutic agent arrived in February 2013, when the FDA announced the approval of Kadcyla®, for the treatment of metastatic breast cancer.[57]

Adcetris® consists of the bio-vector brentuximab (IgG1 cAC10), which is a chimeric monoclonal antibody, to target the human CD30 antigen on B-cells.[58] The antibody is attached to a combination linker, *via* the Cysteine Sulfhydryl (Cys-SH) groups. These are generated from the mild reduction of the inter-chain hinge disulfide bonds of the antibody.[59] This linker combination is made up of a thiol-reactive maleimidocaproyl (mc) spacer, the dipeptide Valine-Citrulline (Val-Cit) linker and a 4-aminobenzylcarbamate (PABC) self-immolative spacer.[60] This set-up facilitates the conjugation of on average four drug Molecules of Monomethyl Auristatin E (MMAE) on the antibody (Figure 4).[61] MMAE is so toxic to healthy cells that it cannot be used as a stand-alone chemotherapeutic.[62]

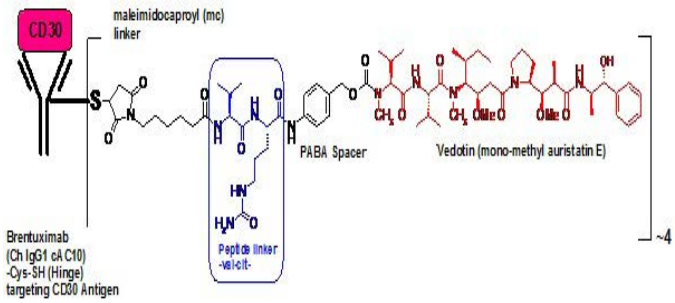

Figure 4: Structure of Adcetris®

In the mechamism of action ADC binds to the antigen on the B-cell to from an ADC-antigen complex (Figure 5). The ADC-antigen complex in the case of Adcetris® is internalized by clathrin-mediated endocytosis and transported to the intracellular lysosome compartment. The ADC-antigen complex fuses with the lysosome and the action of cathepsin-B proteases initiates a spontaneous intramolecular [1,6]-elimination of PABC to release the free-drug MMAE (picomolar potency) into the cytoplasm.[63] This drug then inhibits microtubule assembly, causing depolymerization, leading to cell cycle arrest which results in cell death.[64]

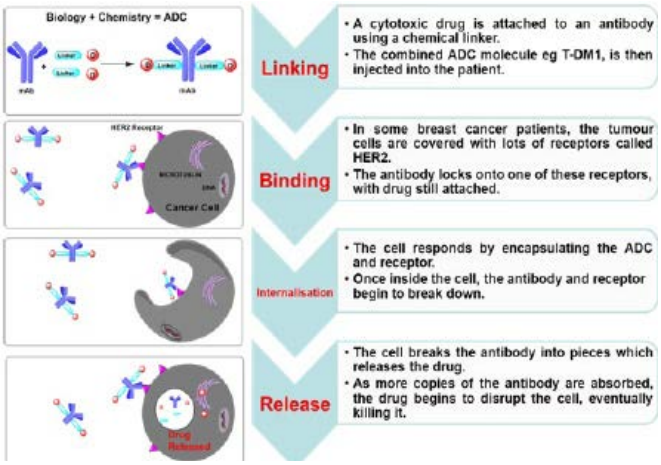

Figure 5: Mechanism of Action of ADCs

Kadcyla® consists of three components (Figure 6): the humanized MAb (IgG1) trastuzumab (Herceptin®) to target HER2 tumour antigens; the microtubule polymerization inhibitormaytansinoid DM1 drug and the (N-Maleimidomethyl) Cyclohexane-1-Carboxylate (MCC) non-cleavable thioether linker. Once the ADC is internalised into the cancer cell it undergoes catabolic metabolism releasing the cytotoxic drug DM1 from the antibody.[65]

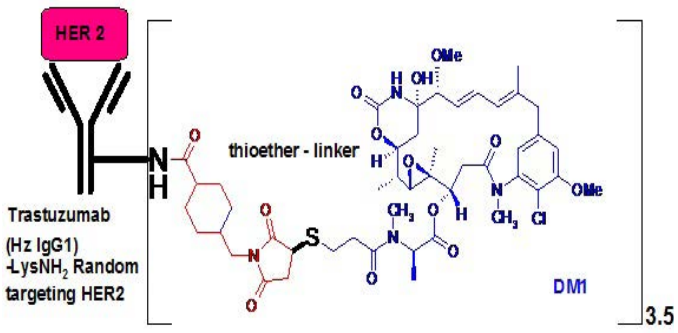

Figure 6: Structure of Kadcyla®

The majority of ADCs contain a number of the same drug attached to the monoclonal antibody, thereby producing heterogeneous mixtures. Kadcyla®, exists in such a heterogeneous form, ranging from 0-9 DM1 drug-molecules on each monoclonal antibody, with an average of 3.5 DM1 molecules per monoclonal antibody.[66] The tumour killing action of DM1, is in the inhibition of cell division, by binding tubulin, arresting the target cell in the G2/M stage of the cell cycle which results in apoptosis.[67]

Currently, strategies are being developed to produce ADCs with a greater degree of homogeneity. This is particularly directed to the Drug to Antibody Ratio (DAR), to circumvent regulatory issues.[68] The majority of ADCs typically contain a binomial distribution of cytotoxic drugs per monoclonal

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antibody, typically varying from 0-8 drugs moieties per ADC molecule. These emerging technologies are able to influence and aid the homogeneity of the DAR ratio.[69]

FUTURE PROSPECTS

Targeting cancer cells with specific monoclonal antibodies which carry cytotoxic drugs and radionuclide payloads is now a reality. This was first envisaged by Paul Ehrlich over 100 years ago. The main aim of this approach is to limit the damage to surrounding healthy cells in the vicinity of tumour cells. Currently, over 130 patients have received this experimental cancer treatment which is called Targeted Alpha Therapy (TAT). Information gathered from these first clinical trials will contribute to future safety profiles for the administration of alpha emitters in future patients.

The real successes have come from Bexxar® used in the treatment of non-Hodgkin's lymphoma, by delivering beta and gamma radiation from iodine-131. Conversely, the radiopharmaceutical Zevalin® is used both as an SPECT imaging agent and also as a therapeutic bullet. The destruction of the cancer is achieved by the usage of the beta emitter yttrium-90 to target and destroy B-cell non-Hodgkin's lymphoma.

At present, the biopharmaceutical industry is excited by the FDA approvals of Adcetris® to treat Hodgkin's lymphoma and Kadcyla® for the treatment of metastatic breast cancer. The only alpha particle emitting radioactive therapeutic agent approved by the FDA is Xofigo®, for the treatment of castration-resistant prostate cancer. This advancement of medical imaging techniques will deliver greater success to the targeted therapy approach in the management and treatment of oncological disease states.

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Corresponding author

Razi Vago, PhD

Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of The Negev, P.O.Box. 653, Beer Sheva, 84105, Israel

Tel. +927-86479087

Fax: +927-86477196

E-mail: rvago@bgu.ac.il

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Effects of Three Dimensional Microenvironment on Tumorigenicity of Fibrosarcoma *in vitro*

Irit Levinger, Racheli Zagouri, Yvonne Ventura and Razi Vago*

Avram and Stella Goldstein-Goren, Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

ABSTRACT

Tumor microenvironment plays an important role in cancer progression owing to interactions between the tumor and adjoining cells and, as in bone marrow, the unique architecture and chemical compounds that characterize it. It has recently been proposed that bone marrow shelters cancer cells in niches which may favor regulation of their quiescence, although the mechanisms involved have yet to be elucidated. We conjectured that a bone marrow mimicking, calcium providing 3D biolattice recently developed by our group could affect fibrosarcoma cells in various ways, whether *via* its specific architecture or due to its provision of calcium, an element correlated with many tumorgenic processes. In order to verify our conjecture, we examined the modifications induced in fibrosarcoma cells by this biolattice. We found that its regulatory effects on fibrosarcomas enhanced tumorigenicity, mediated by up-regulated tumorigenesis related genes. We observed decreased proliferation of cancer cells accompanied by up-regulation of genes associated with cancer stem cells, pointing to a process of de-differentiation. In addition, our results revealed up-regulation of Wnt4 and c-Myc in cells cultured on the biolattice, along with down-regulation of AXIN-1 and WIF-1. Taken together these findings suggest that a calcium rich bone marrow-like microenvironment can affect the tumorigenic capacity and fibrosarcoma cells de-differentiation through the mediation of the Wnt signaling pathway.

KEYWORDS: Microenvironment; Tumorigenicity; Fibrosarcoma.

INTRODUCTION

In the last few years numerous studies have demonstrated the important part played by the tumor microenvironment in the regulation of cancer development and progression, both *in vitro* and *in vivo*.[1-4] It was shown that the microenvironment has a crucial impact on the tumorgenic potential of cultured cancer cells.[5,6] Kaplan et al. (2005) demonstrated that a distant tissue-specific milieu had the capacity to initiate tumor cell migration towards pre-metastatic sites, emphasizing the role of the microenvironment in cancer progression *in vivo*.

A variety of factors in the microenvironment have been proposed as causatives triggering cancer progression, such as Extracellular Matrix (ECM) signals/receptors,[6,7] the mechanical properties of the immediate surroundings of the tumor,[8] and calcium.[2,9,10,11] Calcium also influences tumor cells *via* calcium dependent molecules.[9,12,13]

Many studies highlight the importance of calcium in regulating cancer development and progression.[2,9,11,12] Although calcium signaling and triggering mechanisms have not been proven to be a prerequisite for cancer formation, they are associated, either directly or indirectly, with each of the known cancer hallmarks[14,15] and cancer related features.[2,4,11,12,16,17]

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Metastases are formed when tumor cells lose their cell-cell adhesion and are free to migrate to secondary tumor sites. Cell-cell adhesion is mediated by calcium dependent transmembrane adhesion molecules of the cadherin super family. Cadherins enable interactions between different cell types in a variety of organisms through homophilic binding.[18,19] Cancer cell migration to secondary tumor sites is also regulated by cadherin alterations *via* Epithelial-Mesenchymal Transition (EMT).[18,20] The cadherin intracellular region is attached to β-catenin, which acts in convergence with the cadherin adhesion mechanism and canonical Wnt signaling pathways.[21,22] Decrease in cadherin levels may promote tumorgenesis not only by causing immediate cancer cells looseness and therefore enhanced migration capacity, but also by serving as a positive regulator of Wnt signaling, leaving unbound cytoplasmic β-catenin free to reach the nucleus. When it reaches the nucleus β-catenin acts as a transcriptional co-activator of the TCF/LEF family of transcription factors, triggering the transcription of target genes, one of which is the c-Myc oncogene. c-Myc has many downstream target genes that regulate cancer cell proliferation and migration, as well as many other tumorgenic capacities.[21,23-26] Wnt pathway constitutive activation is commonly observed in carcinomas, and recently upregulation of the Wnt/β-catenin cascade was demonstrated in sarcomas model and correlated with sarcoma aggressive growth and metastasis.[27-29]

Although the importance of cadherin-mediated cell adhesion and Wnt signaling for malignant processes has been extensively discussed;[13,18] the role of calcium in the tumor microenvironment and its correlation with the relevant signaling pathways and with cancer progression are not yet fully understood.

Bone Marrow (BM) is a calcium microenvironment located in cancellous bone, the spongy, soft inner part of long bones. The inorganic phase of BM, which consists mainly of $Ca_5(PO_4)_3(OH)$ (hydroxylapatite), is characterized by a large surface area and high porosity.[30] It is highly vascularized and comprises many cell types, including fibroblasts, hematopoietic cells, adipocytes, osteoblasts, osteoclasts, and Mesenchymal Stem Cells (MSCs).[30-32]

For culturing cancer cells, as earlier studies have demonstrated, 3D models are to be preferred to the traditional monolayers.[33] Their 3D architecture offers cancer cells the possibility of arraying themselves in structures that, mimicking the *in vivo* environment, promote native cell-cell communications, cell-matrix interactions, and ECM secretion.[34]

Bone marrow attracts homing tumor cells,[1,35] providing them with a calcium rich shelter.[36,37] In light of the proven involvement of calcium in enhanced malignancy capacity, it seems likely that BM induces changes in the tumorigenicity of Cancer Cells (CCs). To test this hypothesis, it would be necessary to study tumor related processes in an adequate 3D model with a calcium rich milieu. Although current three-dimensional models offer good mimicry of certain *in vivo* tumor surroundings[8,38-40] to date no model providing optimal 3D mimicry of the calcium rich bone marrow microenvironment has been described.[1,39]

To circumvent this difficulty, we have developed a novel 3D model that mimics the BM microenvironment in regard to its chemical and physical properties as well as its ability to provide calcium in a direct and indirect manner. The biolattice chosen for the present research is a skeletal derivative of a marine invertebrate, the coral *Porites lutea* (POR),which is composed of $CaCO_3$ in the crystalline form of aragonite. It has already been demonstrated that, owing to the significant similarity between this biolattice and the inorganic domain of bone, POR is a suitable microenvironment for culturing MSCs and inducing their osteogenic differentiation.[41,42]

METHODS

EXPERIMENTAL DESIGN, SCAFFOLD AND CELLS

Experimental design

Four different cell culture combinations were used in this study. Scaffolds consisting of aragonite from the skeleton of the coral *Porites lutea* (POR) were designated as the 3D group. To enable comparison of the surface characteristics of the bioactive scaffold with those of an inert matrix, a second group of POR scaffolds was coated with gold; this was designated 3Dg.

To provide a two-dimensional negative control group, cells were grown on a plastic surface designated 2D. Finally, cells were grown on a plastic surface in the presence of POR matrix, which was placed in an insert above the cell culture, to provide a two-dimensional positive control group; that group was designated 2D-CM (2D conditioned medium).

Scaffolds

Scaffolds from *Porites lutea* (POR) were cut into blocks and polished to a thickness of approximately 0.5 mm and an area of 0.5 cm^2 using a grinder (model South Bay Technology 900-8). To remove organic residues, the samples were bleached with commercial hypochlorite solution, rinsed with distilled water, and dried in air. Scaffolds were autoclaved (121ºC, 30 min). To produce the gold-covered scaffolds, polished POR samples were placed in a Veeco VE, VP-776 evaporator and coated first with a 100 Å chromium layer, then with a 300 Å gold layer under a constant chamber pressure of 10^{-6} Torr at a rate of 3 Å/s. The 3Dg matrices were rinsed with 70% ethanol, then with distilled water, and autoclaved (121ºC, 30 min).

Cell culturing and seeding

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Mus musculus (BALB/C) fibrosarcomas were seeded on plastic dishes or on POR scaffolds in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L D-glucose, 1 mM sodium pyruvate, 10% (v/v) Fetal Bovine Serum (FBS), 1% L-Glutamine, and 1% Pen-Strep-Neomycin solution (all from Biological Industries, Israel). Cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced every 2-3 days.

Murine MSCs (ATCC/CRL 12424), were differentiated towards endothelial like cells using differentiation medium (modified DMEM with 2% FBS, 50 ng/mL Vascular Endothelial Growth Factor-A (VEGF), 25 ng/mL basic-Fibroblast Growth Factor (bFGF) [both from PeproTech, Israel], 10 units/mL Heparin sodium salt [Sigma, Israel] for four days. Differentiated cells were seeded on POR scaffolds and 2D control monolayers in order to demonstrate the lack of acquiring tumorigenic related features of non-tumorigenic cells growing in the POR microenvironment.

MICROSCOPY

Light microscopy

Cell morphology and quantity were imaged using a light inverted phase-contrast microscope (Eclipse Ti, Nikon) fitted with a digital camera (D5-Qi1Mc, Nikon).

Scanning Electron Microscopy (SEM)

A stock solution of fixative was prepared from 2% paraformaldehyde in distilled water and 2.5% glutaraldehyde in 0.1M phosphate buffer. Samples were rinsed with Phosphate Buffered Saline (PBS) (Biological Industries, Israel) and immersed in the fixative at 37ºC for 30 min. After fixation, the samples were washed three times with PBS for 10 min, then soaked in serial gradients of 50%, 75%, 90% and 95% ethanol for 10 min each and three times in 100% analytical ethanol for 10 min each. The samples were then immersed in Hexamethyldisilazane (HMDS) (Bel-Gar, Israel) and ethanol solutions in three different volume ratios (1:2, 1:1 and 2:1). Samples were dried in a hood overnight. The morphology of the samples was examined with a SEM (JEOL, JSM-5610-LV).

HISTOCHEMICAL ANALYSIS - SAFRANIN O STAINING

On day 7 of the culture period, samples were examined for glycosaminoglycan content.

Safranin O stains proteoglycans and glycosaminoglycans, known components of cartilage/ECM tissue. A stock solution of 1% Safranin O/Fast Green FCF (Sigma; 0.5 g in 100 ml of isopropanol) was prepared. Samples were rinsed three times with PBS, fixed with 12% formaldehyde for 5 min and then stained with Safranin O/Fast Green FCF stock solution for 15 min. Serial ethanol gradients (50%, 75%, 90%, 95%, and twice 100%) were used to rinse the samples. The 2D samples were photographed with a light inverted phase-contrast microscope (Eclipse Ti, Nikon) fitted with a digital camera (D5-Qi1Mc, Nikon), while the 3D groups were photographed with a stereoscope (SMZ 1500, Nikon), fitted with a digital camera (DXM1200, Nikon).

CELL PROLIFERATION ANALYSIS

Quantification of cells in relation to various treatment procedures was performed using the XTT Cell Proliferation Kit (Biology Industries, Israel). The assay is based on the ability of metabolically active cells to reduce the tetrazolium salt XTT to orange colored compounds of formazan; the dye formed can then be read at 490 nm with a spectrophotometer. Prior to the test, a calibration curve was prepared from samples with various known cell numbers. The samples were incubated with XTT reagent for 4 hours, after which the intensity of the dye formed measured with a spectrophotometer (SynergyMx, BioTek, USA). Cell number was calculated according to the calibration curve.

MIGRATION ASSAY

It was demonstrated that MSCs attract cancer cells .[43-45] This MSCs feature was used in a migration assay, where MSCs serve as inducer and attractant for cancer cell migration.

Mesenchymal stem cells (ATCC, CRL 12424) were seeded at the bottom of 12-well plates. Inserts (Greiner Bio-One ThinCert, 8 µm pore size) were placed on top of the wells, forming a migration chamber. Cancer cells belonging to all four different cell culture groups (2D, 2D-CM, 3D, 3Dg) were seeded into the inserts: monolayers for the 2D and 2D-CM groups; scaffolds with seeded cells were placed in the inserts - for the 3D and 3Dg groups. Cells were allowed to migrate for 7 days, after which they were collected from the bottom zone of the inserts and counted. Parallel to the migration assay, the cell quantity in the inner part of the insert was determined for calculation of a number of migrated cells per 1000 cells present.

QUANTITATIVE GENES EXPRESSION MEASUREMENTS

RNA extraction

Total RNA was extracted from the cells using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Intact RNA integrity was tested on 1.7% agarose gel containing 0.0025% (v/v) ethidium bromide. The concentration and purity was determined at 230 and 260 nm (NanoDrop ND-1000, USA).

Complementary DNA

cDNA was synthesized from total RNA using ran-

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dom hexamers with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. The reactions were incubated in a thermal cycler for 10 min at 25ºC, 120 min at 37ºC, 5 min at 85ºC and then held at 4ºC.

qPCR

The sources of the gene-specific primers (Table 1) and their nucleotide sequences are summarized in Table 1 together with the quantity of cDNA used for each gene analysis (determined according to a standard curve at the accepted value after 25 cycles (Ct ~ 25)).

Gene	Design	Accession number	Forward and reverse primers
TBP	Primer Express® software (V3.0, Applied Biosystems, USA)	NM_013684.3	F: 5'-CCCCTATCACTCCTGCCACAC-3' R: 5'-AAGTAGACAGACTGAAGCTGCGGTA-3'
c-Myc	Primer-BLAST (NCBI)	NM_010849.4	F: 5'-AAACCCCGCAGACAGCCACG-3' R: 5'-TCGGCGGTGGAGAAGTTGCC-3'
SOX2	IDT validated primer	NM_011443.3	F: 5'-GTACAACTCCATGACCAGCTC-3' R: 5'-CTTGACCACAGAGCCCAT-3'
S100A4	IDT validated primer	NM_011311.2	F: 5'-AGCTTCATCTGTCCTTTTCCC-3' R: 5'-TGTAATTGTGTCCACCTTCCAC-3'
CCL2	IDT validated primer	NM_011333.3	F: 5'-TGATCCTCTTGTAGCTCTCCA-3' R: 5'-CATCCACGTGTTGGCTCA-3'
CCL5	IDT validated primer	NM_013653.3	F: 5'-CCTCTATCCTAGCTCATCTCCA-3' R: 5'-CAAGTGCTCCAATCTTGCAG-3'
E-cadherin	Primer-BLAST (NCBI)	NM_009864.2	F: 5'-ACCAGTTTCCTCGTCGCGC-3' R: 5'- TTCAGAGGCAGGGTCGCGGT-3'
N-cadherin	Primer-BLAST (NCBI)	NM_009733.2	F: 5'-GACAAAGAAACCCAGGAAAAGTGGC-3' R: 5'-CTCTCTTCTGCCTTTGTAGAGCTCC-3'
AXIN1	Primer Express® software (V3.0, Applied Biosystems, USA)	NM_009733.2	F: 5'-GTCCAGTGATGCTGACACGCT-3' R: 5'- GCCCATTGACTTGGATACTCT-3'
WIF1	Primer Express® software (V3.0, Applied Biosystems, USA)	NM_011915.2	F: 5'-CTTTGCTGGCAACAGTGCCTC-3' R: 5'-GGTCCTAAGGATGGTGTTGCC-3'
Wnt4	Primer Express® software (V3.0, Applied Biosystems, USA)	NM_009523.2	F: 5'-GAAGGTGGTGACACAAGGGA-3' R: 5'-CTGCAAAGGCCACACCTG-3'
CD44	Primer Express® software (V3.0, Applied Biosystems, USA)	NM_001039151.1	F: 5'-CCTCAGCCCCTCCTGAAGA-3' R: 5'-CGAGTACCATCACGGTTGCAC-3'

Table 1: Primers for quantitative real time polymerase chain reaction (qPCR) analysis of gene expression.

For each reaction 10 µL of mixture containing 5 µL SYBR® Green PCR Master Mix (Applied Biosystems, USA), 4 µL of cDNA, primers (Table 1) and complete amount of double distilled water (treated with diethyl pyrocarbonate (DEPC) was prepared. qPCR reactions were performed using an Applied Biosystems Step One Plus qPCR. The reactions were incubated in a 96-well plate at 95ºC for 20 min followed by 40 cycles of 3 secs at 95ºC and 30 sec at 60ºC and finally 15 min at 95ºC for the melt curve. ΔΔCT with TATA-box Binding Protein (TBP) as housekeeping gene was calculated.

All analyses were performed at least in triplicate, and three independent experiments were performed.

STATISTICAL ANALYSIS

Each series of experiments included at least three or more independent biological repeats and at least three technical replicates. The results were expressed as means ± standard error. Student t-test was performed and statistical significance was determined at a value of $p<0.05$ for each experiment.

RESULTS

PROLIFERATION RATE

Cancer cells demonstrated a decreased proliferation rate on POR 3D scaffolds as compared with 2D scaffolds (Figure 1a). The influence of calcium on proliferation became evident when cell counts were compared for gold coated versus non-coated scaffolds - the presence of calcium caused a decrease in proliferation rate (Figure 1b). Despite the imperfect surface coverage of the 3Dg scaffolds during the gold coating process, the calcium induced decline in cell count was more pronounced in the case of the 3D platforms.

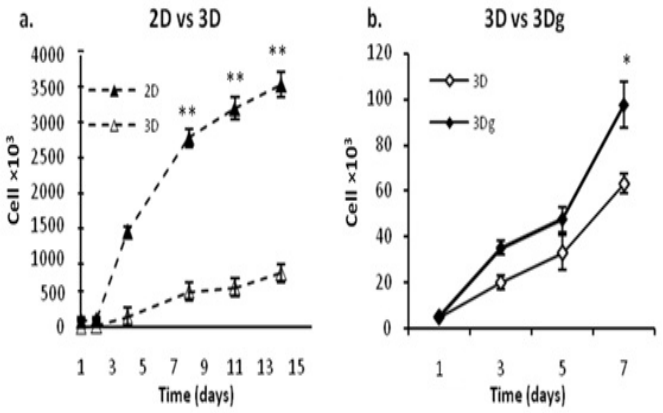

Figure 1: Fibrosarcomas proliferation rate a: 3D scaffolds *vs.* 2D control group. b: 3Dg *vs.* 3D group. Asterisks denote significant differences between treatments at the same time point; *P<0.05, **P <0.01

MIGRATION

Since proliferation rate - one of the key features of cancerous cell cultures - was clearly affected by the POR microenvironment, we decided to check whether the latter also influenced the ability of the cancer cells to migrate. In light of our group's earlier studies in which MSCs demonstrated spontaneous differentiation towards an osteoblastic fate when cultivated on the POR biolattice,[41] we speculated that restrained proliferation could point to differentiation and hence restricted movement and tumorgenic functioning. However, this notion was dispelled by our further results: on both 2D surfaces and 3D POR scaffolds, cells grown in the presence of calcium displayed enhanced migration abilities (Figure 2a). Compared with the 2D control group, taken as base line, migration ability on the 2D surfaces supplemented with POR conditioned medium (2D-CM) was 3.2 times higher (Figure 2b). Among the 3D groups the differential was smaller - cell migration numbers recorded in non-coated matrices were only 1.95 times higher (Figure 2b), possibly because of incomplete sealing of

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the plated matrix (the thin layer of gold only coated the outer surface of the scaffolds, leaving exposed surfaces of $CaCO_3$ inside the pores).

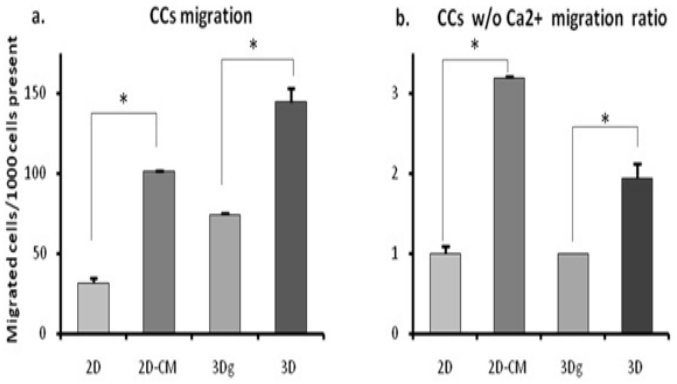

Figure 2: Fibrosarcomas (CCs) migration: a: Migrated cells/1000 cells present b: Migration ratio between groups with/without calcium. Asterisks denote significant differences between treatments; *P<0.05, **P <0.01

CELL DISTRIBUTION AND TISSUE DEVELOPMENT ON POR

Fibrosarcoma cells were seeded on the biomatrices, coated and non-coated. The cells adhered and proliferated. After seven days of culturing cancer cells had covered the inside of the matrix pores, and after 15 days they had formed tissue-like structures inside the pores and covered other matrix areas as well (Figure 3). On the 3D biomatrices the cells looked more dense and volumetric than on 2D-CM (see Section ECM secretion). Many cell-cell and cell-scaffold interactions were detected.

On the gold coated scaffolds the cells adhered mostly to the inner surfaces of the pores (Figure 3a), which were only partially coated and thus presented exposed calcium, indicating cell preference for calcium providing sites.

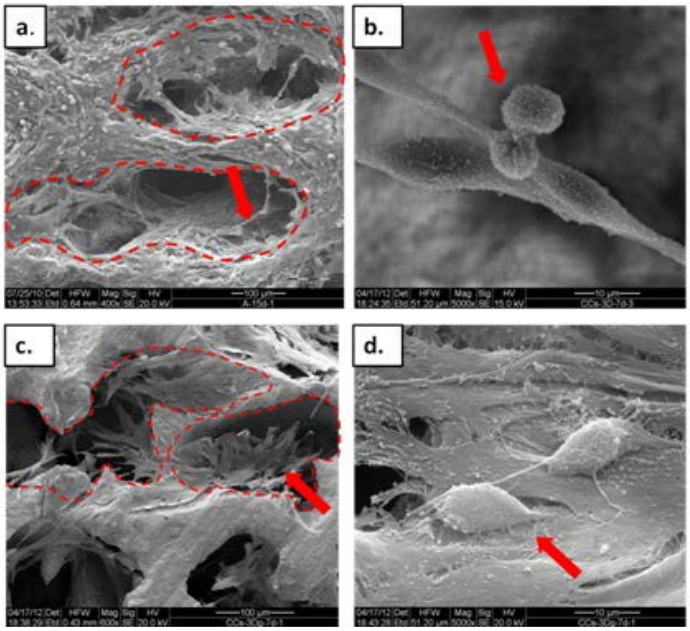

Figure 3: SEM of fibrosarcomas on the POR scaffolds: a-b: 3D POR, ×400 and ×5000, respectively c-d: gold coated-3Dg POR, ×600 and ×5000, respectively. The dashed lines indicate the pore edges of the scaffolds and arrows point to cell aggregates/cells.

ECM SECRETION

Cells grown on POR matrices built an interconnected network, but also streamed down to the plate surface. In the 2D cultures near the scaffolds an altered morphology was demonstrated in areas adjoining the pieces of POR scaffold - cell aggregates could be distinguished (Figure 4). The cells in these aggregates seemed to be tightly packed and were volumetrically shaped, recalling SEM images of the POR cultures. Their appearance suggests that calcium from the matrices had affected the secretion of ECM in these cells. Safranin-O staining confirmed the presence of glycoaminoglycans (GAGs), testifying to elevated secretion by the aggregated cells in the vicinity of the POR pieces (Figure 4b).

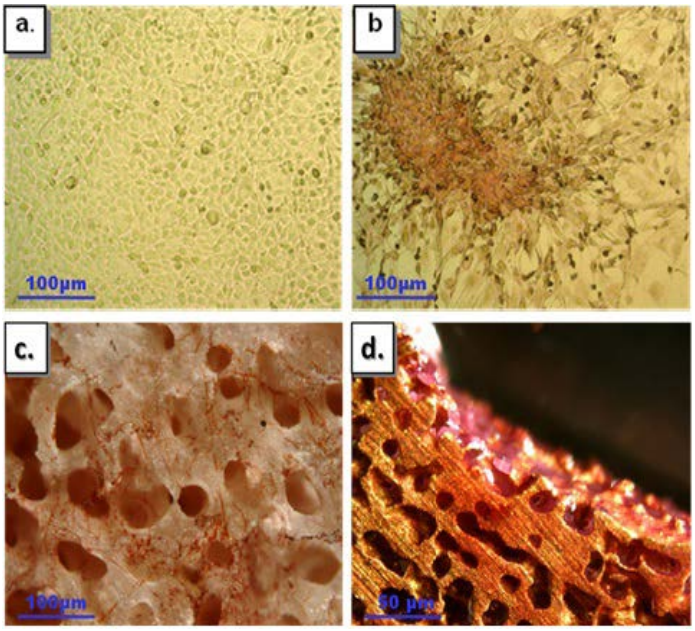

Figure 4: Safranin-O/Fast Green staining of fibrosarcomas (a-b: light microscopy, c-d: binocular) a: 2D control. b: cell aggregates near POR scaffold, on plate surface. c: 3D POR, cells adhered to all scaffold surface d: 3Dg POR, cells adhere only to surface with exposed calcium areas mainly found on the inner sides of the scaffold.

Fibrosarcoma cells on the POR scaffold demonstrated ECM secretion as well: cells were seeded on both gold coated and non-coated scaffolds, and after 7 days of culturing they were treated with Safranin O/Fast Green (Figure 4c-d). Cells on non-coated matrices were stained red, indicating ECM secretion (Figure 4c), while on gold coated matrices cells demonstrated Safranin O red staining mainly in the gold-free areas, where the $CaCO_3$ crystals of the native POR scaffold were exposed (Figure 4d). Formed tissues are more easily distinguished along the edges of the scaffold, along with clear evidence of ECM secretion (data not shown).

TUMORIGENIC-RELATED GENES mRNA LEVELS

In order to shed light on the changes that occurred in the tumorgenic capacities of the fibrosarcomas cultured on our model, we evaluated the expression of several tumorgenic and metastasis related markers, namely CCL2 and CCL5, which are related to tumor progression and metastasis,[46-49] and S100A4,[12,13] a calcium dependent protein product associated

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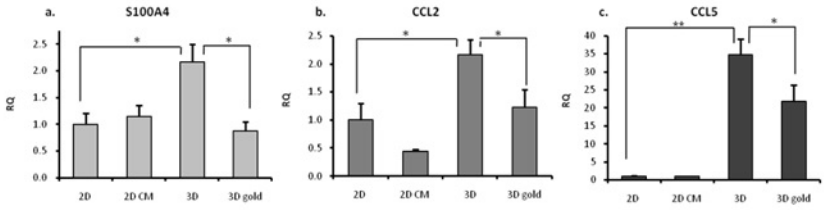

Figure 5: qPCR - tumorgenic-related genes mRNA expression levels (RQ was calculated in relation to 2D control): a. S100A4 b. CCL2 c. CCL5. Asterisks denote significant differences between treatments; *P<0.05 **P <0.01

with enhanced migration capacity and tumor aggressiveness[12,13,50] (Figure 5). For all three markers the highest expression levels were recorded in the 3D group. In the case of CCL5, expression levels detected in the 3D and 3Dg groups were respectively 35 and 22 times higher than in the 2D control (Figure 5c).

Since the Wnt pathway is known to play an important role in decision-making processes in cancer cells, we examined the expression levels of key molecules involved in this pathway. As shown in Figure 6a, elevated expression of Wnt4, one of the Frizzled ligands, was recorded in the 3Dg and especially in the 3D group, and c-Myc oncogene, a Wnt pathway target gene (Figure 6a). Expression of Wnt pathway inhibitors WIF1 and AXIN1 - the latter is a core protein in the GSK3β-β-catenin-APC destruction complex which prevents β-catenin from traveling to the nucleus by binding it to the complex - was inversely correlated to c-Myc expression in the 3D group only (Figure 6b).

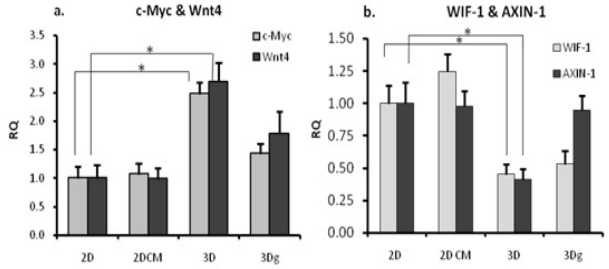

Figure 6: qPCR- Wnt pathway genes mRNA expression levels. RQ was calculated in relation to 2D control: a. Wnt4, c-Myc b. AXIN-1, WIF-1. Asterisks denote significant differences between treatments; *P<0.05

Non-tumorigenic mesenchymal stem cells differentiated towards an endothelial fate, were cultured on POR scaffold and mRNA levels of CCL5 and c-Myc were determined. Results (Figure 7) showed that these cells did not demonstrate up-regulation of the tumorigenic-related genes.

Cells in the control (2D) and experimental groups (2D-CM, 3D, 3Dg) demonstrated an altered cell arrangement and distinct adhesion patterns, implying diversity in the level of expression of the adhesion molecules. E-cadherin and N-cadherin levels were checked and found to be inversely related: high N-cadherin levels were accompanied by low E cadherins level in the 3D group, and *vice versa* in the 3Dg group (Figure 8); N-cadherin levels in the latter group were three times lower than in the 3D group (Figure 8a).

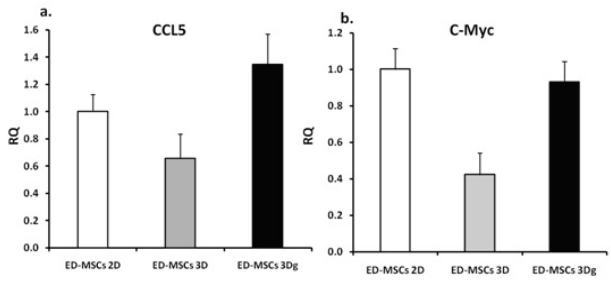

Figure 7: qPCR - endothelial differentiated cells (MSCs origin) mRNA expression levels ofa. CCL5 and b. c-Myc. Cells were cultured on 2D control, 3D and 3Dg POR and show no significant differences between the tested groups

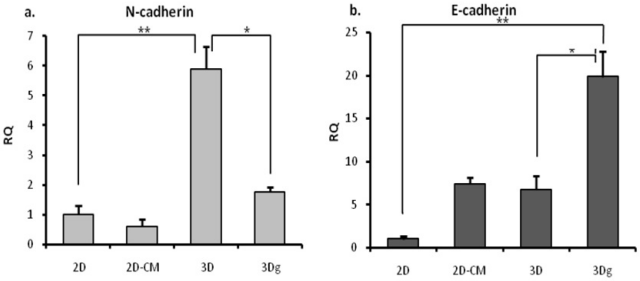

Figure 8: qPCR - E/N cadherin mRNA expression levels: RQ was calculated in relation to 2D control: a. N-cadherin b. E-cadherin. Asterisks denote significant differences between treatments; *P<0.05, **P <0.01

FIBROSARCOMA CELLS STEMNESS-RELATED GENES mRNA LEVELS

Since differentiation may be a causative factor in decreased proliferation and malignancy,[51] and inspired by our previous work with MSCs on POR scaffolds - where MSCs demonstrated spontaneous differentiation towards osteoblasts in the absence of added growth factors[41,52] – we explored this possibility with reference to our cells. However, the fibrosarcomas did not exhibit over-expression of osteogenic-related markers such as RunX and collagen type I (data not shown).

Decreased proliferation may also result from the converse of differentiation, namely stem cell-like state and associated proliferation quiescence. Restrained proliferation rate, increased migration capacity, over-expression of tumor-related markers - taken together these appear to suggest evolution of the cancer cells in the direction of greater stemness.

Two markers related to cancer cell stemness were tested: SOX2[53-55] and CD44, which is also associated with tumor aggressiveness.[56-58,59] Expression of both these genes was found to be up-regulated in the 3D and 3Dg groups (Figure 9).

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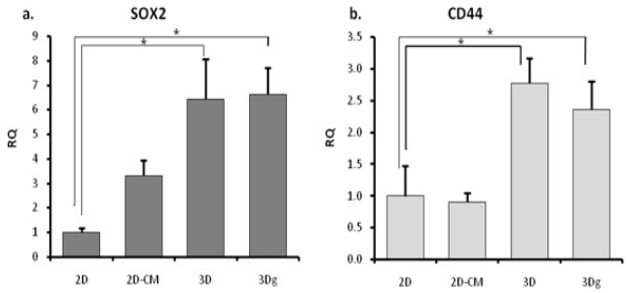

Figure 9: qPCR - cancer stem cells related genes expression: RQ was calculated in relation to 2D control: a. SOX2 b. CD44. Asterisks denote significant differences between treatments; *P<0.05, **P <0.01

DISCUSSION

Using the bone marrow-like calcium providing POR biolattice as a model, we were able to demonstrate that the microenvironment is capable of modifying the tumorigenic capacity of fibrosarcoma cells, and more particularly that it can affect key features - their proliferation rate. Cells grown on the 3D scaffolds proliferated at rates that were five times slower (Figure 1) than those in the 2D group. Supporting earlier findings regarding cancer cell tumorigenicity in bone marrow,[36,60] cells cultured on the calcium rich POR biolattice showed elevated tumorigenic capacity, in keeping with their elevated migration potential (Figure 2) and the up-regulated mRNA levels of several genes associated with tumor progression and metastasis (CCL2, CCL5, and the calcium dependent S100A4). Furthermore, our data indicate that the calcium rich POR biolattice triggers up-regulation in CSCs-related genes mRNA level, which may indicate acquisition of characteristics associated with cancer stem cells (Figure 9).

Previously, the POR biolattice was proven to induce spontaneous MSCs differentiation towards osteblasts.[41,50] However, the same microenvironment induced tumor promoting effects in the case of a fibrosarcomas, such as increased migration (Figure 2) and elevated tumor-related genes CCL5 and c-Myc (Figure 5); this phenomena were not evident in the non-tumorigenic cells (Figure 7), indicating that the tumorigenic effect of the microenvironment is limited to malignant cells.

Bone marrow has been described as a reservoir of Cancer Stem Cells (CSCs);[61-63,64] however, it remains to be established whether cancer cells reach their bone marrow niches already equipped with upgraded tumorigenic capacities, or whether their tumorgenic potential may be regulated by this unique microenvironment. Our results suggest that a bone marrow-like microenvironment is capable of inducing changes in fibrosarcoma cells migration capacity, aside with up-regulation of genes, which are correlated to cancer increased aggressiveness and de-differentiation. CD44 and SOX2 genes have been reported to be closely linked to cancer stemness. Elevated expression of SOX2 has been shown to be dependent on cell environment, and it is followed by cell de-differentiation and the appearance of tumor-initiating stem cell phenotypes.[53] Aside from being correlated with a cancerous stem cell state and extended invasiveness abilities,[64] CD44 has been proven to be a pivotal factor in homing and engraftment of cancer cells in their quiescence niche in bone marrow.[56-58,59]

Cancer stem cells may remain quiescent in the BM niche for long periods,[36] migrating to a future tumor site and establishing new malignancy even years after the primary tumor was eliminated.[1,36,37] In our study, cancer cells cultured on 3D POR biolattice showed upgraded tumorigenic capacity, as manifested in their elevated migration potential towards MSCs, which served as a migration trigger (Figure 2). We suggest that the calcium providing milieu may induce a cancer stem cell-like state, endowing cells cultivated on POR scaffolds with greater tumorgenic potential.

In addition to these changes, we demonstrated that fibrosarcoma cells exploited the scaffolds' unique architecture to construct tissue-like clusters. Similarly, cells in monolayer culture in the conditioned medium or in close proximity to the biolattice underwent changes in morphology and migration capacity. Our results also reveal increased ECM secretion in cells grown on and near POR scaffold (Figure 4).

Calcium signals have recently been shown to affect Epithelial-Mesenchymal Transition (EMT) induction, suggesting a possible avenue of research for the therapy of metastases.[10] The fact that EMT is associated with changes in the expression of the calcium-dependent cadherins[18,65] further highlights the pivotal role of calcium in neoplastic progression. The cadherin switch is thought to be correlated with both EMT and cancer cell aggressiveness and metastatic capacity.[20,66]

In our study just such a transition may have taken place on the non-coated POR scaffolds (Figure 8). The group with the highest tumorgenic abilities - those cells grown on POR (3D) - exhibited the highest N-cadherin mRNA expression (about three times higher than the 3Dg group), whereas their E-cadherin mRNA levels were three times lower. In the 3Dg group the architecture of the scaffolds favored multiple E-cadherin mediated cell-cell interactions, with the gold coating at least partially blocking the access to calcium. In the 3D group, on the contrary, the free availability of calcium from the 3D surface enabled up-regulation of N-cadherin accompanied by down-regulation of E-cadherin.

Alterations in E-cadherin level can also serve as a positive regulatory factor for Wnt signaling *via* E-cadherin-β-catenin release. In conformity with this finding, the group that displayed enhanced migration capacity (3D) (Figure 2a) along with elevation in genes related to increased tumorigenicity (Figure 5) also demonstrated heightened expression of Wnt4 and c-Myc (Figure 6a). Up-regulation of these genes was accompanied by down-regulation of the Wnt pathway inhibitors WIF-1 and AXIN-1 in

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3D group (Figure 6b). These results suggest Wnt pathway involvement in the processes which led to upgrading of the tumorgenic capacity in cells grown on POR biolattice. Involvement of the Wnt pathway in the acquisition of advanced tumor features by fibrosarcoma cells is in agreement with the literature, where the Wnt pathway is characterized as a prominent force controlling cell proliferation, differentiation, and apoptosis[21,25,27,29] Besides Wnt, other cancer related pathways and markers, such as the ERK/MAPK pathway and Ras, have also been shown to be regulated by calcium concentration.[9] The calcium dependence of these pathways bears witness to the particular importance of this ion for malignant processes.

Based on these findings, we propose a model in which a calcium rich, bone marrow-like microenvironment activates certain processes involving Wnt signaling, which lead to an increase in the tumorigenic capacity and de-differentiation of fibrosarcoma cells.

Via the convergence effect of the Wnt-Cadherins pathways, the bone marrow-like biolattice influences decision-making pathways in cancer cells seeded onto the scaffold. On the one hand the three-dimensional architecture supports tissue-like cell organization with multiple cell-cell interactions, and on the other hand, the ample availability of calcium ions triggers changes in the expression of calcium dependent molecules. The combined effects of the calcium providing milieu result in a shift in the cancer cells' differentiation state and enhanced tumorigenic abilities (Figures 6 and 9).

In light of our findings, we further suggest that when circulating tumor cells reach the bone marrow they switch to a quiescence mode, which shelters them until such time as some cues turns on proliferation and motility mechanisms. During their stay in the bone marrow the cancer cells are driven towards a more stem-like state on the stemness-differentiation continuum by the bone marrow microenvironment or/and by neighboring cells such as MSCs. We also suggest that acquisition of stem cell-like properties is an essential precondition for tumorigenicity enhancement, and that these cancer cells may have a superior capacity to invade a blood vessel and migrate towards the future site of a secondary malignancy, causing metastasis.

The POR calcium providing biolattice shows a resemblance to bone marrow pre-metastatic niches in terms of architecture, chemical composition, and a biological effect on cancer cells. Furthermore, our results suggest that the POR biolattice enhances the tumorgenic character of cultured fibrosarcomas, indicating that the BM-like microenvironment possesses the ability to interfere with cancer cell signaling pathways, mediating an enhanced tumorigenic potential and de-differentiation.

The up-regulation of cancer stemness-related genes and elevated tumorgenic potential observed on this biolattice prove enrollment of the calcium providing milieu in the acquisition of higher tumorigenic capacities by seeded fibrosarcoma cells. The POR model for cancer cell culturing brings us one step closer to understanding the complexity of the interactions occurring inside pre-metastatic niche of bone marrow and the microenvironmental influences affecting the cancer cells which it harbors. However, additional research and models are needed for better understanding the role of extra cellular calcium with conjunction of the tumor microenvironment and its role in controlling tumor development at the cellular and molecular level.

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Review

Corresponding author
Nikolai A. Timchenko. PhD
Division of Pediatric Surgery
Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue
Cincinnati, OH, 45229, USA
Tel. 513-636-0129
E-mail: Nikolai.Timchenko@cchmc.org

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Elimination of Tumor Suppressor Proteins during Liver Carcinogenesis

Nikolai A. Timchenko[1*] and Kyle Lewis[2]

[1]Division of Pediatric Surgery, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH, 45229, USA

[2]Department of Pathology and Huffington Center of Aging, Baylor College of Medicine, TX, Houston, 77030, USA

ABSTRACT

Liver cancer is one of the most lethal cancers. Quiescent liver expresses up to 20 tumor suppressor proteins including Rb, p53, CCAAT-Enhancer-Binding Protein (C/EBP)α, Hepatocyte Nuclear Factor (HNF4)α and p16 and it is well protected from development of liver cancer. However, the negative control of liver proliferation by these factors and other tumor suppressor genes is eliminated in liver cancer. Studies of liver regeneration after surgery and injury have provided fundamental mechanisms on how liver neutralizes tumor suppressor proteins for the time of regeneration; however, studies of liver cancer in animal models and in human samples showed several additional pathways of this neutralization. One of these additional pathways includes activation of a small subunit of the proteasome, Gankyrin. Gankyrin is dramatically increased in human hepatocellular carcinoma (HCC) and in animal models of carcinogenesis. Once activated Gankyrin triggers degradation of main tumor suppressor proteins during development of liver cancer using slightly different mechanisms. Recent studies identified mechanisms which repress Gankyrin in quiescent livers and mechanisms of activation of Gankyrin in liver cancer. These mechanisms involve a communication between Farnesoid X Receptor (FXR) signaling and chromatin remodelling proteins mediated by members of C/EBP family. It has been recently shown that C/EBPα plays a critical role in this network and that the activation of C/EBPα in cirrhotic livers with HCC inhibits cancer progression. This C/EBPα-dependent inhibition of liver cancer involves activation of a majority of tumor suppressor genes and repression of tumor initiating pathways such as β-catenin and c-myc. These recent findings provide a background for FXR-based and C/EBPα-based approaches to treat liver cancer.

KEYWORDS: Liver cancer; Tumor suppressor genes; Gankyrin; C/EBPA; Rb, p53; HNF4α.

INTRODUCTION

The development of hepatocellular carcinoma (HCC) has a long history of affecting mainly adults. In the majority of cases, HCC develops in patients which have chronic liver diseases and/or are under chemical treatments. These chronic diseases affect many signaling pathways leading to liver cancer. One of the critical events in the development of HCC is the loss of hepatocytes to properly control proliferation mainly associated with inability of hepatocytes to stop proliferation. This failure to terminate liver proliferation in HCC patients is associated with the reduction or neutralization of a negative control of liver proliferation. In this review, we summarize recent publications which provide new insight into mechanisms of termination of liver proliferation under normal conditions when liver proliferates but does not develop liver cancer and recent reports that show how these mechanisms of termination are eliminated during development of HCC leading to continued proliferation and tumor growth. Mechanisms of

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normal liver proliferation/termination have been investigated in several models including liver proliferation/termination during postnatal development, liver proliferation/termination after surgical resections (partial hepatectomy) and liver proliferation after acute treatments with carbon tetrachloride (CCl4). These systems provided general principles of termination of liver proliferation under conditions when liver does not develop cancer. Investigations of liver cancer in animal models were mainly focused on the development of liver cancer after treatments with diethylnitrosamine (DEN), while fewer studies have been done with the chronic treatments by CCl4.

PARTIAL HEPATECTOMY AS A MODEL FOR THE STUDY OF MECHANISMS WHICH TERMINATE LIVER PROLIFERATION

One of the key characteristics of liver cancer is uncontrolled liver proliferation. It is well recognized that malignant cells lose the ability to stop proliferation. The understanding of mechanisms which stop liver proliferation is important for development of therapeutic approaches to treat liver cancer. One of the best systems for the studies of mechanisms that terminate liver proliferation is Partial Hepatectomy (PH). The most common model of PH involves resections of 2/3 of the liver which leads to initiation of liver proliferation and restoration of the original size. While mechanisms of initiation of liver proliferation after PH are well investigated and are described in several recent reviews,[1-6] very little is known about the mechanisms that terminate liver regeneration. Global gene profiling of the liver 3 weeks after PH has identified alterations in cell cycle, apoptosis, TGFβ and angiogenesis signaling.[7] PPAR signaling and lipid metabolism have also been implicated in the termination of liver regeneration.[8] It has been shown that certain micro RNAs may be involved in the termination of liver regeneration.[9,10] In addition, the ablation of integrin-linked kinase leads to enhanced liver proliferation.[11] A recent paper by Koral et al. have shown that leukocyte-specific protein (LPS) serves as a tumor suppressor and inhibits proliferation of hepatoma cell lines.[12] It has been shown that termination of liver regeneration after PH and after liver injury requires a tight cooperation of chromatin remodeling proteins and a family of C/EBP proteins and that disorganization of this cooperation leads to a failure of the liver to stop regeneration.[13] A number of key regulators of liver biology are under control of C/EBP family proteins and are properly regulated during liver development, differentiation and regeneration. These proteins include SIRT1, PGC1α, p53, FXR, TERT, enzymes of glucose metabolism PEPCK, G6Phase, Glut2 and Glut4 as well as enzymes of triglyceride syntheses.[14-18] The ability of C/EBP proteins to activate or repress these genes depends on their association with p300 or with HDAC1. Using specific knock-in animal models, Jin et al. found that these known targets are mis-regulated in the liver if the C/EBP-chromatin remodeling complexes are not controlled in a proper way which leads to the lack of termination of liver regeneration.[13,19] Among additional candidates for the termination of liver proliferation, Yap (Yes-associated protein) has been implicated in the regulation of tissue growth and size.[20] It has been shown that Yap protein is activated in the liver after surgical resections and in hepatocellular carcinoma.[21,22] The expression of Yap is under tight control of Hippo signaling which is also changed after PH and in hepatocellular carcinoma.[22] Most important, Yimlamai et al. have shown that Hippo-Yap pathway is critical for maintenance of differentiation state of hepatocytes.[23] In summary, studies of liver regeneration after PH have identified several candidates which might terminate liver proliferation, but are eliminated by liver cancer. Although these studies are important and useful for understanding of mechanisms of liver cancer, it has become clear that development of liver cancer includes several additional pathways to block termination of proliferation. In this review, we focus on the mechanisms by which liver cancer eliminates liver-specific tumor suppressor proteins.

LIVER SPECIFIC TUMOR SUPPRESSOR GENES

The quiescent status of the liver is supported by many Tumor Suppressor Genes (TSG). It has been shown that the activity of more than 20 different TSGs is lost in HCC due to mutations or due to hyper-methylation of their promoters.[24] The TSGs include micro-RNAs which behave as tumor suppressors.[25-27] Epigenetic control is also involved in support of TSGs as it has been shown by genome-wide methylation analysis.[28,29] Further studies provided convincing evidence that many of these TSGs are involved in the protection of liver from development of cancer. Detailed information for these tumor suppressor genes of the liver has been discussed in several recent reviews.[24,30] Therefore, we will here briefly discuss some of these TSGs which are related to the focus of our review. One of the important TSGs is Deleted in Liver Cancer (DLC1) tumor suppressor gene. This gene is located on chromosome 8p22 and plays a critical role in multiple liver functions. It has been shown that DLC1 is deleted in 40% human HCC[31,32] and that restoration of its expression resulted in inhibition of liver proliferation and reduction of the development of tumors after xenografting HCC cells into nude mice.[33] Exomic sequencing of hepatitis C virus (HCV)-associated HCCs has identified novel mutations in AT-Rich Interactive Domain 2 (ARID2) protein which has been further shown to be a liver tumor suppressor protein.[34] A family of Suppressors of Cytokine Signaling (SOCS), are inhibitors of cytokine signaling. It has been shown that the liver specific deletion of a member of this family, SOCS3, leads to the increased liver proliferation and formation of hepatocellular carcinoma.[35] Among more than 20 known tumor suppressor proteins of the liver, Rb, p53, HNF4α C/EBPα and p16, are investigated in great detail and have been shown to be most critical inhibitors of liver proliferation.

TUMOR SUPPRESSOR PROTEIN P53

P53 is a transcription factor which regulates expression of many genes by direct binding to their promoters.[36] Under conditions when liver is challenged by surgical resections or treatments with drugs, expression of p53 is elevated

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leading to growth arrest, induction of apoptosis, or senescence.[37,38] It has been also shown that p53 regulates ploidy of hepatocytes. Using p53 KO mice, Barton's group has shown that ploidy levels increased during regeneration of both Wild-Type (WT) and p53(-/-) hepatocytes, but only WT hepatocytes were able to dynamically resolve ploidy levels and return to normal by the end of regeneration. Kurrina et al. identified multiple cell cycle and mitotic regulators (Foxm1, Aurka, Lats2, Plk2, and Plk4) as direct targets of p53 in the liver.[37] The expression and activity of p53 is significantly reduced in the majority of cancers including hepatocellular carcinoma.[39,40] In about 50% of patients with HCC, the reduction of p53 levels and activity is mediated by mutations within the coding region or within the p53 promoter.[40] However, a number of recent studies revealed that the elimination of p53 by ubiquitin proteasome system contributes to the loss of p53 tumor suppressor functions in cancers.[41] The main ligase that triggers p53 degradation is MDM2 which targets six key lysine amino acids on p53.[42] In addition to MDM2, there are other ligases that target p53 degradation such as CHIP (C-terminus of HSP70 interaction protein).[41,43] It is interesting that MDM2 is a transcriptional target for p53 which creates an auto regulation loop that works under conditions of DNA damage. The DNA damage stabilizes p53 protein, but it is degraded by MDM2-proteasome pathway by activation of its own inhibitor at the time when cells recover after stress and do not need p53 anymore.[44-46] The MDM2-dependent degradation of p53 involves other proteins which cooperate with MDM2[47] or control levels of MDM2. This review is focused on the one of these regulators, Gankyrin, which stabilizes MDM2 and facilitates degradation of p53 during development of liver cancer (see below).

P16/RB/E2F PATHWAY IN LIVER PROLIFERATION AFTER PH AND IN LIVER CANCER

Cell cycle progression in proliferating livers is stimulated by E2F transcription factors which activate several key S-phase specific genes.[4] The E2F family consists of eight members, five of which (E1F1-E2F5) interact with Rb, while E2F6-E2F8 do not and work as a repressor of E2F-dependent genes. It has been shown that E2F1 plays an overlapping role in HCC[48] and E2F2-E2F7 promote cancer.[49] E2F8 transcription factor is a unique member of the family which represses promoters without interactions with Rb. It has been shown that inactivation of both Rb and E2F8 works synergistically to trigger DNA replication.[50] In addition, E2F8 is essential for polyploidization in mammalian cells.[51] The detailed information for the role of E2F family in cancer has been described in a recent review.[49] Similar to other quiescent tissues, the activity of E2F transcription factors is inhibited in quiescent livers by retinoblastoma, (Rb) protein. Among several members of E2F family, E2F2 seems to be a most important regulator of liver proliferation and timely liver regeneration after PH.[52] It is important to emphasize that C/EBPα is one of the critical regulators of Rb-E2F complexes and that aged livers have a weak proliferation after PH due to C/EBPα-mediated enhancement of Rb-E2F repression function.[53,54] C/EBPα also regulates E2F complexes with another member of Rb family, p107, which brings about growth arrest in hepatocytes.[55] Although C/EBPα -mediated regulation of Rb-E2F complexes is involved in the control of liver proliferation, the most significant pathway of regulation of Rb-E2F complexes is associated with cyclin dependent kinases cdk4 and cdk6. Upon stimulation of liver proliferation by surgical resections, cdk4/cdk6 kinases are activated by cyclin D1 and phosphorylate Rb leading to the dissociation of Rb-E2F complexes.[56] The activities of cdk4/6 are negatively regulated by a member of inhibitors of cdk (INK) proteins, p16. Despite numerous studies of p16 in the liver, very little is known about its role in liver proliferation after PH. Lee et al. showed that p16 undergoes methylation after PH which correlated with liver proliferation.[57] Another study of liver proliferation in aged mice revealed that p16 is elevated in livers of old mice and contributes to the weak proliferative response of livers to PH.[58] Studies of 130 old human patients who underwent hepatectomy showed that these patients had much higher levels of p16 and that these levels negatively correlated with liver regeneration.[59]

Examination of mutation/expression of p16 and Rb proteins in human liver cancer and in animal models of carcinogenesis strongly indicated that the loss of functions of these proteins is involved in development of severe liver cancer. It has been shown that p16 is inactivated at early stages of hepatocarcinogenesis.[60] It has been also shown that p16INK4a pathway is altered in rat liver tumors induced by NNK.[61] The inactivation of p16 and Rb in human HCC samples has been shown in many publications which are summarized in several reviews.[62-64] These reviews emphasized that p16, cyclin D1 and Rb pathways are commonly targeted in various cancers. To determine the role of the disruption of these three pathways in HCC, Azichi et al. have analyzed p16, pRB and cyclin D1 in 47 patients with HCCs. The authors have shown that inactivation of p16 was detected in 64% of HCCs; while Rb was inactivated in 28% of HCC samples. Importantly, several patients had inactivation both of these pathways.[65] In this study, over expression of cyclin D1 was detected in 11% of examined samples. These observations showed critical role of p16-Rb pathway in protection of liver from development of cancer. In agreement with these observations, Viatour et al. have deleted three members of Rb family (Rb, p107 and p130) and found that these triple knockout mice develop liver cancer with gene expression profile similar to that of human HCC.[66] Further studies from this group revealed that Hippo pathway is activated at later stages in these mice.[67]

C/EBPα: A STRONG INHIBITOR OF LIVER PROLIFERATION AND A TUMOR SUPPRESSOR PROTEIN

C/EBPα belongs to the C/EBP family of proteins, βZIP proteins which contain basic region and luecine zipper region.[4,68] These proteins are transcription factors which dimerize with each other and control multiple functions in different tissues.

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Numerous studies revealed that C/EBPα is a strong inhibitor of liver proliferation.[69-74] Despite the fact that C/EBPα is a transcription factor, its activities are regulated on the levels of protein-protein interactions and post-translational modifications. Growth inhibitory activity of C/EBPα is tightly regulated in the liver. One of the critical pathways that control the growth inhibitory activity of C/EBPα is phosphorylation at Ser193. It has been shown that ph-S193 isoform of C/EBPα is a strong growth inhibitory protein, while un-ph-193 isoform has reduced activity to inhibit liver proliferation.[75-77] Generation of C/EBPα knockin models with substitution of Ser193 to Ala (S193A) and to Asp (S193D) further confirmed the critical role of modifications of S193 in the biological functions of C/EBPα.[13,14,15-18] While liver proliferation after PH is almost completely inhibited in S193D mice, the S193A mice showed an early entry in cell cycle and lack of termination of proliferation after surgeries.[13,15] The tumor suppression activity of C/EBPα has been demonstrated in several animal models. Tan et al. have generated C/EBPα knockin mice in which C/EBPα is expressed from the alpha-fetoprotein promoter (which is active in HCC) and have shown that the elevated expression of C/EBPα inhibits liver carcinogenesis.[74] Examination of liver cancer in C/EBPα S193D mice under conditions of DEN-mediated carcinogenesis revealed that C/EBPα is a critical tumor suppressor protein because its degradation by Gankyrin causes early development of liver cancer.[15] A recent paper by Habib's group showed that activation of C/EBPα in cirrhotic livers with HCC inhibits liver cancer.[78] Regarding levels of C/EBPα in human cancer; C/EBPα was also examined in several reports of human HCC. Examination of levels of C/EBPα in liver tumor sections and non-tumor sections of the same patients has found a significant reduction of C/EBPα mRNA in tumor sections.[79] It has been also shown that the reduced expression of C/EBPα in hepatocellular carcinoma is associated with advanced tumor stage and with shortened patient survival.[80] In addition to transcriptional down-regulation of C/EBPα and degradation of the protein, liver cancer neutralizes the activity of C/EBPα by de-phosphorylation of C/EBPα at S193.[75] Taken together, these studies showed that C/EBPα is a tumor suppression protein and that elimination of growth inhibitory activity of C/EBPα is a critical step in development of liver cancer. C/EBPα- S193D mutant completely inhibits liver proliferation after PH[15] and given this strong growth inhibitory activity of S193D mutant in partial hepatectomy studies, one should assume that these mutant mice should be resistant to the development of liver cancer. However, further studies of DEN-mediated liver cancer in the S193D mice revealed that liver cancer developed a mechanism for complete elimination of C/EBPα by Gankyrin.

LIVER-SPECIFIC TUMOR SUPPRESSOR PROTEIN HNF4α

Hepatocyte nuclear factor 4α (HNF4α), regulates several liver functions including proliferation and differentiation of hepatocytes. HNF4α has been a subject of intensive investigations for almost 20 years. These studies demonstrated that HNF4α is a master regulator of liver biology.[81] In addition to the key role of HNF4α in adult livers; HNF4α is a critical regulator of pre-natal liver development. The studies by Duncan's group revealed that HNF4α controls the development of a hepatic epithelium, liver morphogenesis and the sinusoidal organization of the liver during prenatal liver development.[82, 83] The HNF4α gene contains two promoters, P1 and P2, each produces 6 and 3 HNF4α isoforms correspondingly by alternative splicing.[81] Although the functional relevance of these isoforms is unknown, examination of 450 human colon cancer specimens showed that P1-HNF4α isoforms are lost or localized in the cytoplasm of 80% of examined samples.[84] This paper also showed that phosphorylation of HNF4α by Src tyrosine kinase decreases stability of HNF4α and that this mechanism is likely activated in patients with colon cancer.[84] These observations suggested that HNF4α is involved in protection of cancer. In agreement with these results, the possible role of HNF4α in development of human HCC has been demonstrated by examination of patients with HCC which showed that the expression of HNF4α correlates with epithelial-mesenchymal transition which is involved in metastatic tumor formation.[85] A recent paper by Zhang et al. added additional evidence for the role of reduction of HNF4α in development of HCC.[86] The role of HNF4α in liver cancer was examined in WT mice and in several genetically modified animal models. The studies in mice have shown a critical role of HNF4α in the liver functions of adult animals. These functions include regulation of expression of genes involved in lipid and bile acid synthesis, gluconeogenesis, blood coagulation, differentiation and proliferation. In this review, we focus on the discussion of HNF4α functions in liver proliferation and cancer. Examination of liver biology in acute HNF4α knockout mice demonstrated up-regulation of genes which are associated with liver proliferation and cell cycle control.[87] These studies identified several new direct targets of HNF4α which include Bmp7 and Perp, a regulator of p53-dependent apoptosis. In agreement with these observations, it has been shown that the transient inhibition of HNF4α initiates hepatocellular transformation through microRNA feedback loop circuit.[88] It is interesting that once this circuit is activated, it inhibits expression of HNF4α leading to cancer. Tumor suppressor functions of HNF4α have been demonstrated in rat and mouse livers. Ning et al. have found that HNF4α levels are progressively decreased in the livers of DEN-induced rats and that forced expression of HNF4α blocked development of HCC.[89] The mechanism of this inhibition of liver cancer involves the block of activation of β-catenin signaling. Consistent with this report, Apte's group has shown that hepatocyte-specific deletion of HNF4α in adult mice causes increased hepatocyte proliferation and activation of cell cycle genes.[90] Examination of liver cancer in these hepatocyte-specific knockout mice after DEN injections showed that the deletion of HNF4α significantly increases the number and size of hepatic tumors.[90] While in rat livers HNF4α protected development of liver cancer through inhibition of β-catenin signalling,[89] it appears that in mouse livers HNF4α represses tumor through inhibition of both β-catenin and c-myc expression.[91,92] In the liver, HNF4α is under control of several pathways alterations of which might reduce levels of

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HNF4α and cause liver cancer. One of these pathways is Hippo signaling. Using *in vivo* mouse liver development model, Alder et al. have recently shown that Hippo signaling affects hepatocyte differentiation through HNF4α.[93] It has been also shown that mutations in isocitrate dehydrogenease 1 (IDH1) and IDH2 cause intrahepatic cholangiocarcinoma *via* complete silencing HNF4α and subsequent impaired hepatocyte differentiation.[94]

GANKYRIN: A POWERFUL ACTIVATOR OF LIVER CANCER

As we mentioned above, quiescent livers express more than 20 tumor suppressor genes. How does liver cancer eliminate activity of these TSGs? Examination of early events in the development of liver cancer in chemical models has identified elevation of Gankyrin.[95, 96] Gankyrin (gann-ankyrin repeat protein; gann means cancer in Japanese; also known as p28, p28GANK, PSMD10, and Nas6p) is a non-ATPase subunit of the 26S proteasome and is an oncogene consisting of seven ankyrin repeats that is expressed in several cancer types, particularly HCC in which it was first discovered.[95, 97] Recent studies have shown Gankyrin is up-regulated during initiation and progression of HCC and is correlated with capsular invasion, intrahepatic metastasis, and decreased apoptosis.[95,98,99] Furthermore, siRNA to Gankyrin has been shown to decrease tumor cell growth in nude mice and higher levels of Gankyrin expression have been correlated with poor prognosis in HCC.[100, 101] It has been recently found that the histone deacetylase inhibitor panabinostat (LBH589) inhibits proliferation and metastasis of hepatocellular carcinoma through inhibition of Gankyrin.[101] Li et al. have recently identified microRNA-605 as a potent repressor of Gankyrin which also leads to inhibition of liver cancer.[102] Many studies have investigated the role of Gankyrin in HCC and several pathways have been elucidated. Jiang et al. have shown that Gankyrin is repressed by FXR in quiescent liver and FXR expression is decreased in HCC. This interaction depends on downstream targets of FXR: C/EBPβ and HDAC1, which form a complex to inhibit Gankyrin expression in quiescent tissue.[103] This paper also showed that FXR-mediated prevention of Gankyrin activation in DEN-mediated carcinogenesis inhibits liver cancer.[103] Taken together, these papers clearly demonstrated that the inhibition of Gankyrin leads to inhibition of liver cancer.

MECHANISMS OF GANKYRIN-MEDIATED LIVER CANCER

Investigations of mechanisms by which Gankyrin causes development of HCC showed that Gankyrin has two main cancer-promoting activities. The first activity is associated with the neutralization of at least five tumor suppressor proteins and subsequent support of proteins that promote liver cancer. (Figure 1) summarizes signaling pathways which Gankyrin uses to diminish expression/activities of the tumor suppressor proteins and support high levels of cdk4 and Oct4 which promote liver cancer. It has been shown that Gankyrin binds to MDM2/HDM2 and enhances ubiquitination and degradation of p53.[104]

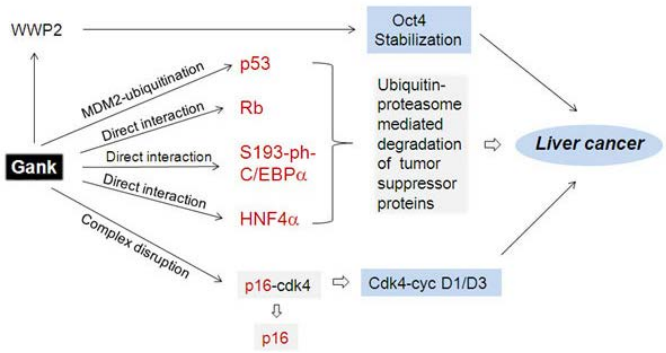

Figure 1: A summary of signaling pathways by which Gankyrin diminishes expression/activities of tumor suppressor proteins and by which it supports high levels/activities of cdk4 and Oct4 promoting liver cancer. Gankyrin directly interacts with C/EBPα, Rb and HNF4α and triggers their degradation. Gankyrin causes degradation of p53 through stabilization of MDM2 ubiquitin ligase. Gankyrin-mediated neutralization of p16 is associated with the disruption of p16-cdk4 complexes and subsequent activation of cdk4 by cyclins D1 and D3. Gankyrin also stabilizes Oct4 by interaction with WWP2 which marks Oct4 degradation.

During the initial discovery of Gankyrin, it was discovered that it is capable of binding Rb through an LXCXE domain and that this leads to increased phosphorylation of Rb and its subsequent degradation.[105] This interaction is involved in conferring anchorage-independent growth in NIH 3T3 fibroblasts. In addition to the interaction with Rb, Gankyrin also binds to D-type kinase, cdk4, and replaces $p16^{INK4a}$ from cdk4 leading to the activation of cdk4.[106] The Gankyrin-mediated elimination of p53, Rb and p16 in liver cancer has been confirmed in many other reports.[2,15,95,103] Recent studies identified two additional targets of Gankyrin; tumor suppressor proteins C/EBPα and HNF4α. As we noted above, C/EBPα is a strong tumor suppressor protein when it is phosphorylated at Ser193. Gankyrin specifically recognizes ph-Ser193 isoform of C/EBPα and S193D mutant and triggers their degradation through the ubiquitin proteasome system. During development of liver cancer in WT mice treated with DEN, C/EBPα is almost completely converted into ph-S193 isoform and becomes a target for Gankyrin.[15] In C/EBPα -S193D mice, Gankyrin eliminates the mutant C/EBPα much earlier leading to fast development of liver cancer.[15,103] Several recent publications from Dr. Wang's group identified HNF4α as additional target of Gankyrin. Using established hepatoma cell lines, this group showed that down-regulation of Gankyrin promotes differentiation of hepatoma cells and that this differentiation is mediated by stabilization of HNF4α. The inverse correlation of Gankyrin and HNF4α was observed in DEN-mediated cancer and in human HCC.[107] In addition to degradation of HNF4α, Gankyrin-dependent dedifferentiation of hepatocytes in tumor initiating cells includes stabilization of Oct4 through Gankyrin competitively binding to WWP2, the ubiquitin ligase that normally marks Oct4 for degradation.[108]

The second liver cancer promotion activity of Gankyrin is associated with activation of signaling pathways which initiate liver cancer. It has been shown that Gankyrin promotes liver tumor growth and metastases through activation of Il-6/STAT3

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signaling.[109] Gankyrin also activates IL-8 during development of liver cancer.[110] Two key pathways of liver cancer, β-catenin and c-myc, are also activated by Gankyrin.[111] In addition, several reports showed that Gankyrin-mediated liver cancer includes activation of PI3K/Akt pathway and Rho/ROCK/PTEN signalling.[112,113] Interestingly, the activation of some of these pathways correlates with expression of stemness factors.[114] Although elevation of Gankyrin in HCC is well documented, very little is known about mechanisms by which liver cancer activates Gankyrin. Our work revealed that Gankyrin is expressed in normal livers at very low levels due to FXR-dependent silencing, but it is activated in liver cancer by the reduction of FXR signalling.[103] FXR supports high levels of chromatin-remodeling complexes C/EBPα-HDAC1 which bind and partially repress the Gankyrin promoter in quiescent liver. Upon treatments with DEN, FXR is reduced leading to de-repression of the promoter.[103] A recent paper suggested an additional mechanism of increase of Gankyrin which is associated with activation of interleukin-1α/IRAK-1 inflammation signaling and subsequent activation of the Gankyrin promoter by NF-Y-p300 complexes.[115] (Figure 2) summarizes current knowledge about activation of Gankyrin in liver cancer and Gankyrin-dependent activities which contribute to development of liver cancer. The activation of Gankyrin in rodent models of carcinogenesis is mediated perhaps by two important events: de-repression of the Gankyrin promoter by reducing FXR signaling and subsequent activation by interleukin-1α/IRAK-1signaling. The elevation of Gankyrin causes elimination of 5 tumor suppressor proteins and activation of positive regulators of cancer such as β-catenin and c-myc. These global alterations contribute to the development of liver cancer.

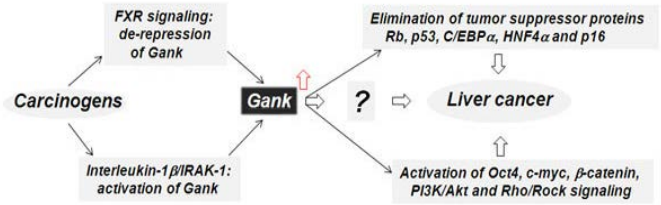

Figure 2: Activation of Gankyrin in liver cancer. Gankyrin is activated by carcinogens using two main pathways: 1) reduction of FXR signaling leading to a release of repression of the Gankyrin promoter; and 2) activation of Interleukin-1α/IRAK-1 pathway and subsequent activation of the Gankyrin promoter by JNK and NF-Y/p300/CBP transcriptional complex. Once activated, Gankyrin displays two main cancer-promoting activities: 1) elimination of tumor suppressor proteins; and 2) activation of tumor-promoting Oct4, c-myc, β-catenin, PI3K-Akt and Rho/ROCK pathways.

TREATMENTS AND PREVENTION OF LIVER CANCER BY INHIBITION OF GANKYRIN AND BY RESTORATION OF ACTIVITIES OF TSGs

Current studies of liver cancer using global profiling of gene expression, chromatin remodeling and proteomics revealed multiple alterations in the liver biology which are associated with each other. This situation suggests that it is unlikely to generate a single-gene therapeutic approach to cure liver cancer. However, literature data also show that Gankyrin is one of the critical components of the development of liver cancer because it controls multiple pathways of liver cancer (Figures 1 and 2). This fact raises a unique possibility to correct/prevent liver cancer by targeting of Gankyrin or by activation of FXR/inhibition of interleukin-1α/IRAK signaling. Among those possibilities, the promising approach might be the activation of FXR because it has been shown that long-lived little mice express high levels of FXR and do not develop liver cancer with age and after treatments with DEN.[103] It has been shown that high levels of FXR prevent activation of Gankyrin and rescue expression of tumor suppressor genes protecting from development of cancer.[103] Moreover, our unpublished results revealed that direct activation of FXR by specific ligand GW4064 rescues tumor suppressor proteins and prevents liver cancer (Lewis and Timchenko, unpublished results).Very promising observations have been recently found in the studies of liver cancer in rat models of cirrhosis and HCC by Habib's group. Using short activating RNA (saRNA) strategy, the authors activated C/EBPα in rats with severe cirrhosis and HCC and found significant inhibition of liver cancer and dramatic improvement of liver functions.[78] Examination of cancer pathways in hepatoma cell lines after activation of C/EBPα by saRNA revealed that correction of C/EBPα expression increased levels of 18 tumor suppressor gene including HNF4α, p53, Rb, DLC1, ARID2 and SOCS3. saRNA- mediated activation of C/EBPα also down-regulated several canonical pathways of liver cancer such as HFG, β-catenin and c-myc signaling. Several critical drivers of liver proliferation were also down-regulated including cyclin D1 and Stat3.[78] Importantly, activation of C/EBPα by saRNA improved liver functions. (Figure 3) summarizes positive effects of activation of C/EBPα in livers with HCC on liver biology and functions.

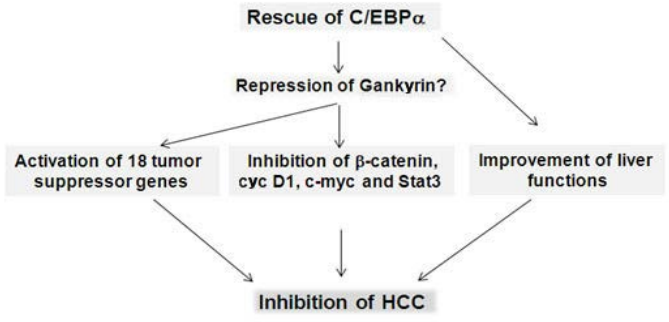

Figure 3: Rescue of C/EBPα expression in HCC inhibits liver cancer. The diagram summarizes observations published in a recent paper[78] and suggests possible mechanisms of C/EBPα -mediated inhibition of liver cancer (see text).

These observations show that C/EBPα is a master regulator of many tumor suppressor genes, critical repressor of tumor promoting pathways, and a positive regulator of liver functions. These observations place C/EBPα in a unique position to be a therapeutic target for the treatments of patients with liver functions. How does the correction of one protein correct so many cancer associated dysfunctions in the liver?

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Although this issue requires further examination of molecular pathways in livers after activation of C/EBPα, literature data and data in our lab suggest some of these pathways such as a possible feedback loop leading to down-regulation of Gankyrin. We have shown that the Gankyrin promoter contains two high affinity C/EBP sites.[103] Therefore, it is possible that activated C/EBPα represses the Gankyrin promoter in complexes with HDAC1 leading to the rescue of TGS and to repression of c-myc and β -catenin signaling (Figure 3). In agreement with this hypothesis, some of the up-regulated TSGs, c-myc and β-catenin are targets of Gankyrin see Figure 2. Regardless of the mechanisms, it is clear that C/EBPα is a key tumor suppressor protein in the liver.

CONCLUSION

Development of liver cancer involves multiple alterations of liver biology on several levels of gene expression complicating development of therapeutic approaches to treat cancer. Although these multiple changes are not easy to correct, recent progress in investigations of tumor suppressor proteins and mechanisms of their elimination in cancer provides a possibility to develop approaches which might reduce liver cancer at advanced stages and improve liver functions. It is likely that tumor suppressor proteins communicate with each other through different signaling pathways and rescue/protection of one of them is sufficient for inhibition of liver cancer. In this regard, tumor suppressor protein C/EBPα is a promising candidate, correction of which inhibits liver cancer. We think that, similar to C/EBPα, correction of HNF4α might also have beneficial effects on the liver since HNF4α regulates liver differentiation and many liver functions. It is also interesting that activities of both these proteins are regulated by specific phosphorylation pathways which also might be considered as possible tools for correction of C/EBPα and HNF4α. However, the most hopeful strategy seems to be activation of their promoters and prevention of their degradation by Gankyrin. Specifically, drug-mediated activation of FXR and subsequent block of Gankyrin elevation could be considered for inhibition of liver cancer in human patients. Some of the known drug-activators of FXR are already in trials for NAFLD and might be quickly incorporated in the trails for patients with HCC.

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