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Bioengineered human tumor within a bone niche

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ABSTRACT

Monolayer cultures of tumor cells and animal studies have tremendously advanced our understanding of cancer biology. However, we often lack animal models for human tumors, and cultured lines of human cells quickly lose their cancer signatures. In recent years, simple 3D models for cancer research have emerged, including cell culture in spheroids and on biomaterial scaffolds. Here we describe a bioengineered model of human Ewing's sarcoma that mimics the native bone tumor niche with high biological fidelity. In this model, cancer cells that have lost their transcriptional profiles after monolayer culture re-express genes related to focal adhesion and cancer pathways. The bioengineered model recovers the original hypoxic and glycolytic tumor phenotype, and enables re-expression of angiogenic and vasculogenic mimicry features that favor tumor adaptation. We propose that differentially expressed genes between the monolayer cell culture and native tumor environment are potential therapeutic targets that can be explored using the bioengineered tumor model.

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1. Introduction

Both the two-dimensional (2D) culture and *in vivo* models of cancer have been actively used to unravel the complex mechanisms and molecular pathways of cancer pathogenesis. Cancer cells lose many of their relevant properties in 2D culture, presumably due to the lack of the native-like physiological milieu with extracellular matrix (ECM), supporting cells and regulatory factors. As a result, 2D cultures are not predictive of antitumoral drug effects in human [1,2]. Animal models have their own limitations in representing human disease [3], necessitating the use of clinical data [4].

Bioengineering methods are just starting to enter the field of cancer research, offering simple 3D models of cancer, such as tumor spheroids, cell inserts, and cell encapsulation in hydrogels or porous scaffolds [5–7]. While these models provide advance over monolayer cultures, cancer cells still remain deprived of native tumor environments providing interactions between cancer cells, stromal and vascular cells [8]. Indeed, Bissel has convincingly demonstrated that the microenvironment can both inhibit and facilitate tumor growth and metastatic [9]. Specifically in the bone microenvironment, it has been shown that osteoblasts, osteoclasts, fibroblasts as well as mesenchymal stem cells (hMSC) play essential roles in primary tumor growth and metastasis [10,11]. However, current approaches are far from replicating the native *in vivo* milieu in which tumors develop, a necessary condition for advancing cancer research and translating novel therapies into clinical practice.

In this report, our aim is to introduce substantial improvements over existing 3D models to study bone tumors by implementing advanced methods in tissue engineering. We have developed a protocol to engineer human bone tumors in their native niche. We cultured Ewing's sarcoma (ES) spheroids within tissue engineered human bone, grown from adult hMSC capable of osteogenic differentiation using a native bone ECM as a structural and mechanical scaffold. This innovative model allows cross-talk between cancer cells and crucial bone microenvironment components, namely osteoblasts, ECM secreted by cells and native mineralized ECM. We propose this novel experimental model as a tool to determine bone tumor targets in a human organ context under conditions predictive of human physiology.

2. Materials and methods

2.1. Native tumors

Fully de-identified samples of Ewing's sarcoma tumors were obtained from the Columbia University Tissue Bank. A total of 44 samples were used in experimental studies. Frozen tissue samples were cut into sets of contiguous 10 μm-thick sections (6 sections per sample) and homogenized in Trizol (Life technologies) for RNA extraction and subsequent gene expression analysis.

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2.2. Retroviral and lentiviral transductions

A GFP line of Ewing sarcoma cells was derived from hMSCs by retroviral transductions performed using an established protocol [12]. The GFP retroviral vector (pBabe-Puro-GFP) was kindly provided by Dr. Manuel Serrano (CNIO, Madrid, Spain) [13]. The EWS-FLI-GFP expression vector was generously provided by Dr. Elizabeth R. Lawlor (University of Michigan, Ann Arbor, MI, USA). Lentiviral transductions were performed following a previously described protocol [14]. EWS-GFP cells were cultured in DMEM supplemented with 10% (v/v) Hyclone FBS and 1% penicillin/streptomycin.

2.3. Tumor cell lines

Ewing's sarcoma cell lines SK-N-MC (HTB-10) and RD-ES (HTB-166) were purchased from the American Type Culture Collection (ATCC). According to the manufacturer's specifications, SK-N-MC cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium (EMEM), and RD-ES cells were cultured in ATCC-formulated RPMI-1640 Medium (RPMI). Both culture media were supplemented with 10% (v/v) Hyclone FBS and 1% penicillin/streptomycin.

U2OS osteosarcoma cell line and HEK293T cell line were kindly provided by Dr. Manuel Serrano (CNIO, Madrid, Spain) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Hyclone FBS and 1% penicillin/streptomycin.

2.4. Cultivation of human mesenchymal stem cells

Cultivation, seeding and osteogenic differentiation of human mesenchymal stem cells (hMSC) were performed as in our previous studies [15,16]. Briefly, hMSC were cultured in basic medium (DMEM supplemented with 10% (v/v) Hyclone FBS and 1% penicillin/streptomycin) for maintenance and expansion, followed by osteogenic medium (basic medium supplemented with 1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate) for osteogenic differentiation. Due to the highly osteogenic properties of the mineralized bone scaffolds used to culture the cells, the supplementation of MBP-2 was not necessary. All cells were cultured at 37°C in a humidified incubator at 5% CO_2.

2.5. Tumor cell spheroids

To form tumor cell spheroids, 0.3×10^6 Ewing's sarcoma cells were centrifugated in 15 mL Falcon tubes (5 min at 1200 rpm), and cultured in 4 mL of medium for one week at 37°C in a humidified incubator at 5% CO_2.

2.6. Tissue engineered model of tumor

Cell culture scaffolds (4 mm diameter × 4 mm high plugs) were prepared from fully decellularized bone as in our previous studies [15,16]. Each scaffold was seeded with 1.5×10^6 hMSCs (passage 3) and cultured in 6 mL of osteogenic medium for 4 weeks. Medium was changed biweekly. After 4 weeks, bone tissue constricts were bisected; one half was infused with aggregates of Ewing's sarcoma cells (3 spheroids per scaffold) (this group was termed ***TE-ES***) and the other half of the bone tissue construct was used as a control (this group was termed ***TE-bone***).

Tumor models were formed using each of the three tumor cell lines (RD-ES, SK-N-MC, EWS-GFP). For each tumor, TE bone without tumor cells was used as a control. ***TE-RD*** model (and their counterpart TE-bone controls) were cultured in RPMI medium; ***TE-SK-N-MC*** model (and their counterpart TE-bone controls) were cultured in EMEM; ***TE-EWS-GFP*** model (and their counterpart TE-bone controls) were cultured in DMEM.

All culture media were supplemented with 10% (v/v) Hyclone FBS and 1% penicillin/streptomycin. TE-ES and TE-bone models were cultured at 37°C in a humidified incubator at 5% CO_2 for 2 and 4 weeks.

2.7. Cytometry

Surface marker analysis by FACS was carried out as described previously [16]. In brief, hMSC and ES cell lines (RD-ES, SK-N-MC and EWS-GFP) were harvested, centrifugated and incubated at 4°C for 1 h with fluorochrome conjugated antibodies APC Mouse anti-human CD13 (BD Pharmingen, 557454), APC Mouse anti-human CD44 (BD Pharmingen, 560532), APC Mouse anti-human CD73 (BD Pharmingen, 560847), APC Mouse anti-human CD90 (BD Pharmingen, 559869) and APC Mouse anti-human CD105 (BD Pharmingen, 562408). Negative control cells were stained with APC mouse IgG1, k isotype control, Clone MOPC-21 (BD Pharmingen, 555751). CD99 expression was assayed incubating cells with CD99 primary antibody (Signet antibodies, SIG-3620). FACS data were analyzed using FlowJo software version 7.6 (Tree Star Inc., Ashland, OR, USA).

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was obtained using Trizol (Life Technologies) following the manufacturer's instructions. RNA preparations (2 μg) were treated with "Ready-to-go you-prime first-strand beads" (GE Healthcare) to generate cDNA. Quantitative real-time PCR was performed using DNA Master SYBR Green I mix (Applied Biosystems). mRNA expression levels were quantified applying the ΔCt method, ΔCt = (Ct of gene of interest − Ct of Actin). GFP primers were selected as previously reported [17]. Other qRT-PCR primer sequences were obtained from the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/) (Table 1).

2.9. Microarray data analysis

Expression of genes in native Ewing's Sarcoma tumors and cell lines was studied in 11 cell lines and 44 tumors by applying the barcode method to the Affymetrix Human Genome U1332 Plus 2 gene expression data of Savola et al. [18]. A probeset was considered expressed only if detected in all cell lines/tumors. Where a gene had multiple probesets, the gene was only counted once. Genes expressed in cell lines, but not tumors, or in tumors, but not cell lines, were identified from the asymmetric differences between the two both sets.

2.10. Histology and immunohistochemistry (IHC)

TE-ES and TE-bone models were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H/E). The sections were then stained for CD99 (dilution 1:500; Signet antibodies, SIG-3620) and GLUT1 (dilution 1:500; Abcam, ab652) as previously described [16], and counterstained with Hematoxylin QS (Vector Labs). For PAS staining, periodic acid-Schiff (PAS) (from Sigma–Aldrich) was used according to the manufacturer's instructions.

Table 1
The list of primers used in qRT-PCR of tumor cells.

Gene description	PrimerBank ID
Beta actin (Actin)	4501885a1
EWS-FLI1 fusion isoform type 8 (EWS-FLI)	633772a1
Homo sapiens NK2 homeobox 2 (NKX2-2)	32307133b1
Homo sapiens tumor protein p53 (TP53)	371502118c1
ACTN4 Homo sapiens actinin, α4 (ACTN4)	316660986c2
CCND2 Homo sapiens cyclin D2 (CCND2)	209969683c1
COL1A2 Homo sapiens collagen, type I, α2 (COL1A2)	48762933c3
COL3A1 Homo sapiens collagen, type III, α1 (COL3A1)	110224482c2
Homo sapiens collagen, type VI, α1 (COL6A1)	87196338c2
COL6A2 Homo sapiens collagen, type VI, α2 (COL6A2)	115527065c1
COL6A3 Homo sapiens collagen, type VI, α3 (COL6A3)	240255534c1
FLNB Homo sapiens filamin B,β (FLNB)	256222414c2
MYLK Homo sapiens myosin light chain kinase (MYLK	116008189c1
Homo sapiens 3-phosphoinositide dependent protein kinase-1 (PDPK1)	60498971c1
Homo sapiens protein phosphatase 1, regulatory subunit 12A (PPP1R12A)	219842213c1
Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1)	163659898c1
VCL Homo sapiens vinculin (VCL)	50593538c1
CDKN1B Homo sapiens cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B)	207113192c3
Homo sapiens C-terminal binding protein 1 (CTBP1)	61743966c2
CTBP2 Homo sapiens C-terminal binding protein 2 (CTBP2)	145580576c1
ETS1 Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	219689117c1
c-K-ras2 protein isoform a (KRAS)	15718763a1
PIAS1 Homo sapiens protein inhibitor of activated STAT, 1 (PIAS1)	7706636c2
Homo sapiens retinoid X receptor, alpha (RXRA)	207028087c3
Homo sapiens signal transducer and activator of transcription 3 (STAT3)	47080104c1
Homo sapiens cell division cycle 42 (GTP binding protein, 25 kDa) (CDC42)	89903014c1
Homo sapiens collagen, type IV, α2 (COL4A2)	116256353c1
Homo sapiens catenin (cadherin-associated protein), β1, 88 kDa (CTNNB1)	148233337c2
Homo sapiens jun proto-oncogene (JUN)	44890066c1
Laminin α4 chain (LAMA4)	4504949a2
Homo sapiens laminin, β1 (LAMB1)	167614503c1
Homo sapiens laminin, γ1 (formerly LAMB2) (LAMC1)	145309325c3
Homo sapiens phosphoinositide-3-kinase, regulatory subunit 1 (α) (PIK3R1)	335057530c3
Homo sapiens phosphatase and tensin homolog (PTEN)	110224474c2
Homo sapiens hypoxiainducible factor 1, α subunit (HIF1A)	194473734c1
Homo sapiens vascular endothelial growth factor A (VEGFA)	NM_001101
Homo sapiens EPH receptor A2 (EPHA2)	296010835c1
Homo sapiens tissue factor pathway inhibitor (TFPI)	98991770c1
Homo sapiens laminin, γ2 (LAMC2)	157419139c1

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