Cell Survival and Tumour growth

Deepak Dinakaran, MD, PhD, MEd
Dept. Medical Biophysics and Radiation Oncology, University of Toronto
Odette Cancer Centre, Sunnybrook Health Sciences Centre

Overview

- Tumour models to study radiation response
- *In vitro* and *in vivo* assays for tumour radiation response
- Cancer Stem cells – potential contribution to radiation response
- Impact of Tumour microenvironment

Learning Objectives

After attending this session, attendees will be able to:

1. Describe the concept of clonogenic survival to assess radiation response

2. Identify *in-vivo* models to assess clonogenic survival

3. Understand cell and tumor microenvironment factors that contribute to radioresistance

Tumour volume doubling time is highly variable

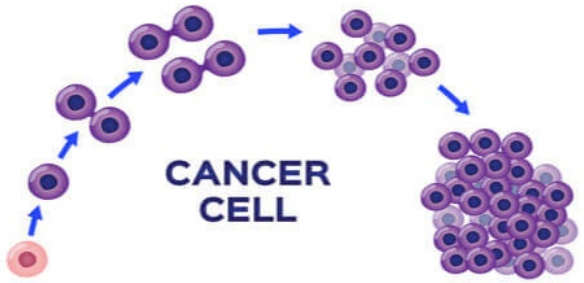


Table 7.1 Volume doubling times (VDTs) for human tumours taken from a review of early data on the growth rate of human tumours

Site and histology	Number of tumours measured	Mean VDT* (days)	Confidence limits (days)
Lung metastases			
Colon–rectum, adenocarcinoma	56	95	84–107
Breast, adenocarcinoma	44	74	56–98
Kidney, adenocarcinoma	14	60	37–98
Thyroid, adenocarcinoma	16	67	44–103
Uterus, adenocarcinoma	15	78	55–111
Head and neck, squamous cell carcinoma	27	57	43–75
Fibrosarcoma	28	65	46–93
Osteosarcoma	34	30	24–38
Teratoma	80	30	25–36
Superficial metastases			
Breast carcinoma	66	19	16–24
Primary tumours			
Lung, adenocarcinoma	64	148	121–181
Lung, squamous cell carcinoma	85	85	75–95
Lung, undifferentiated	55	79	67–93
Colon–rectum	19	632	426–938
Breast	17	96	68–134

*Geometric mean.

Data from Steel (1977).

$$V = \frac{\pi}{6} \times length \times width \times height$$

Time for tumor volume (V) to double

Joiner & van der Kogel.
Basic Clinical Radiobiology 4th ed

Tumour Cell Loss Factors (CLF) greatly influence tumour growth

Table 7.5 Calculation of cell loss factors (CLFs) for human tumours based on labelling with radiolabelled thymidine or thymidine analogues and volume doubling times (VDTs) in separate series

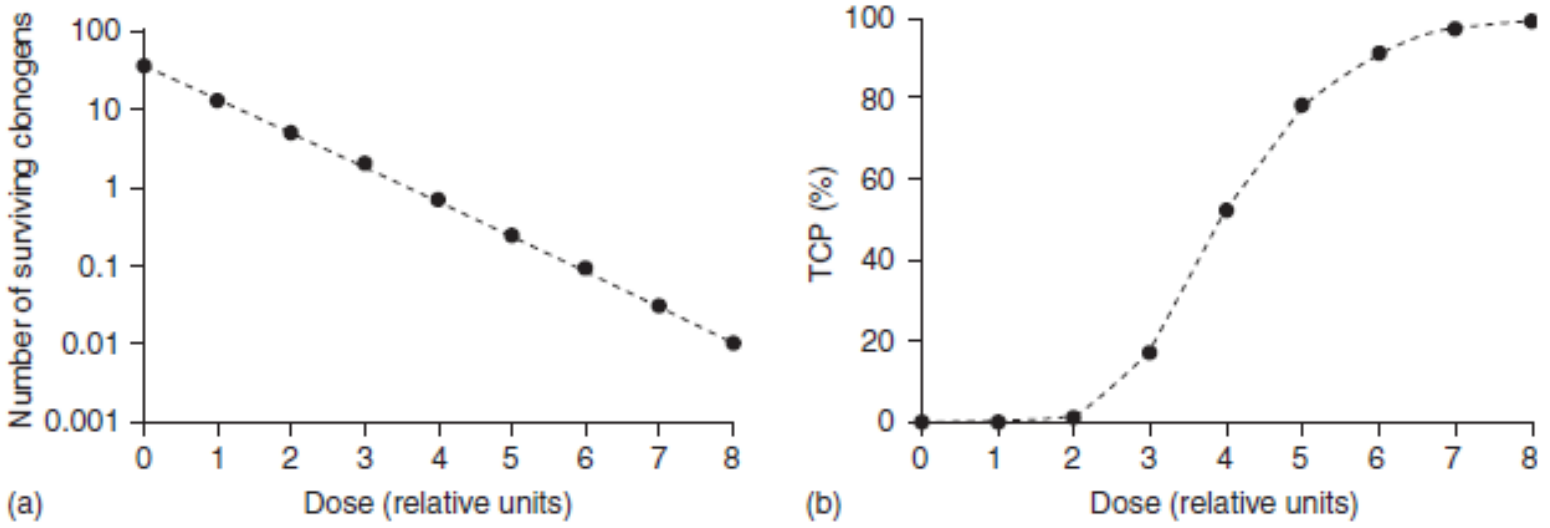
Site	LI (%)	T_{pot} (days)	VDT (days)	CLF (%)
Undifferentiated bronchus carcinoma*,[1]	19.0	2.5	90	97
Sarcoma*,[1]	2.0	23.3	39	40
Childhood tumours*,[1]	13.0	3.6	20	82
Lymphoma*,[1]	3.0	15.6	22	29
Head and neck**,[2]	9.6	4.1	45	91
Colorectal**,[2]	13.1	3.9	90	96
Melanoma**,[2]	4.2	8.5	52	84
Breast**,[2,3]	3.7	9.4	82	89
Prostate**[2,4]	1.4	28.0	1100	97

*,**Labelling with radiolabelled thymidine or thymidine analogues, respectively.
[1]From Steel (1977), calculations assume $T_S = 14$ hours, $\lambda = 0.8$.
[2]Fraction of cells in S phase (LI) and potential doubling time (T_{pot}) from Haustermans *et al.* (1997) and Rew and Wilson (2000); calculations assume $\lambda = 0.8$ (Steel, 1977).
[3]VDT values for pulmonary metastases from Spratt *et al.* (1996).
[4]VDT from PSA doubling times from Schmid *et al.* (1993), Fowler *et al.* (1994) and Lee *et al.* (1995).

- **Reasons for cell loss:**
  - **Quiescent (G0)**
  - **Death (apoptosis, necrosis, etc.)**
  - **Lack of oxygen/nutrients**

Joiner & van der Kogel. Basic Clinical Radiobiology 4th ed

Radiation dose, surviving fraction (SF) and tumour control probability (TCP) – rationale for fractionation



- **Logarithm of surviving clonogenic tumour cells decreases linearly with total radiation dose (left)**
- **For TCP graph (right), note sigmoid curve shape of % tumour control versus dose**

Joiner & van der Kogel. Basic Clinical Radiobiology 4th ed

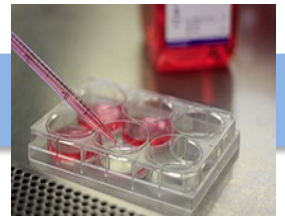
Assays for tumour radiation response

- *In vitro*
  - Radiation clonogenic survival assays
    - Measures *intrinsic radiosensitivity*
    - Note: does not take into account other R's of radiobiology (redistribution, reoxygenation, repair, repopulation)
- *In vivo*
  - *Tumor growth delay*
  - *TCD50*
  - *Ex vivo clonogenic survival*
  - *Clinically: early response biomarkers*
    - **To allow us to better understand the underlying biology of radiation response**
    - **To predict if a treatment combination will be effective**

Clonogenic Survival Assay

Day 1:

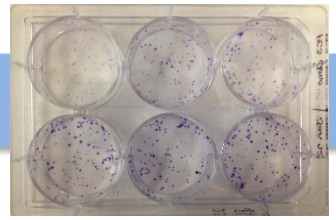
Seed cells



radiation treatment

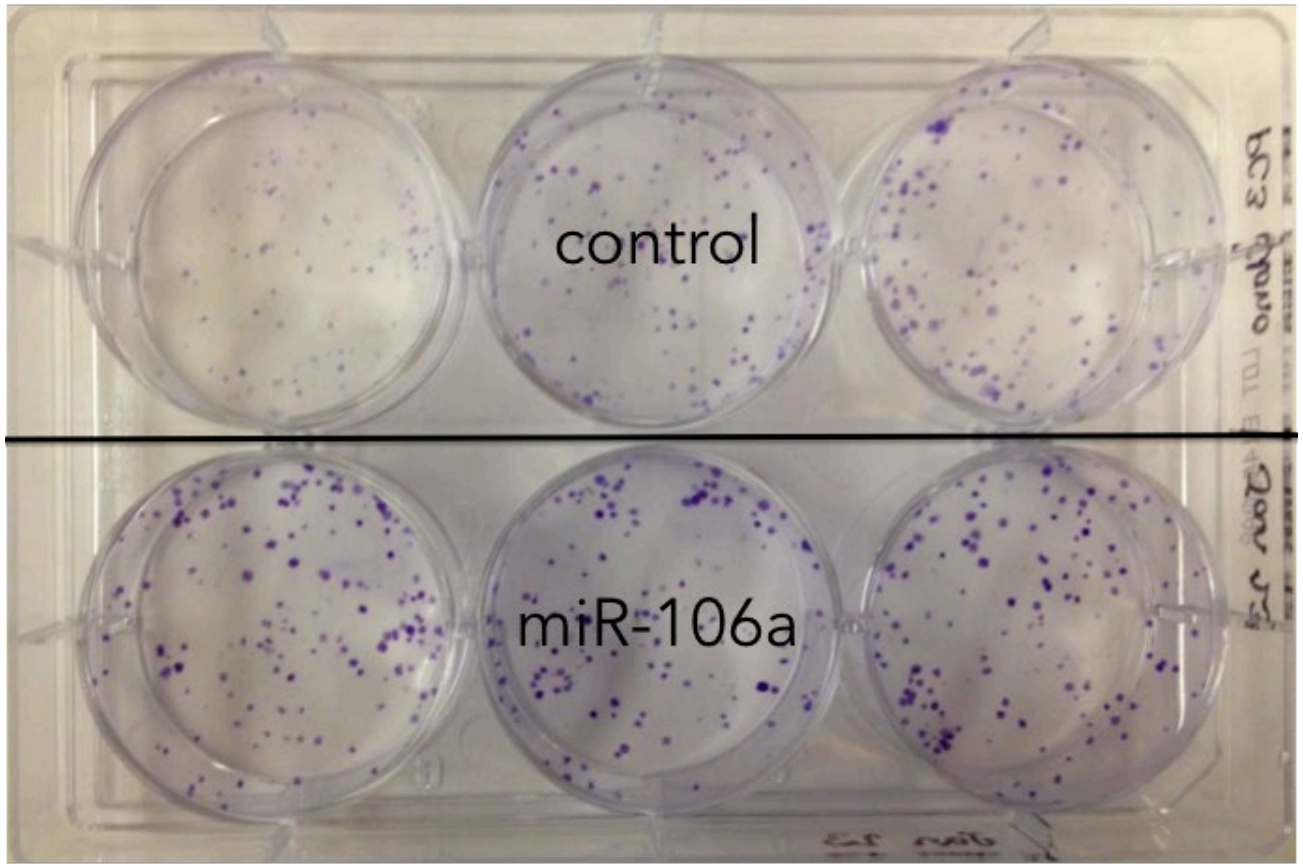
~ Day 10-14:

stain and count



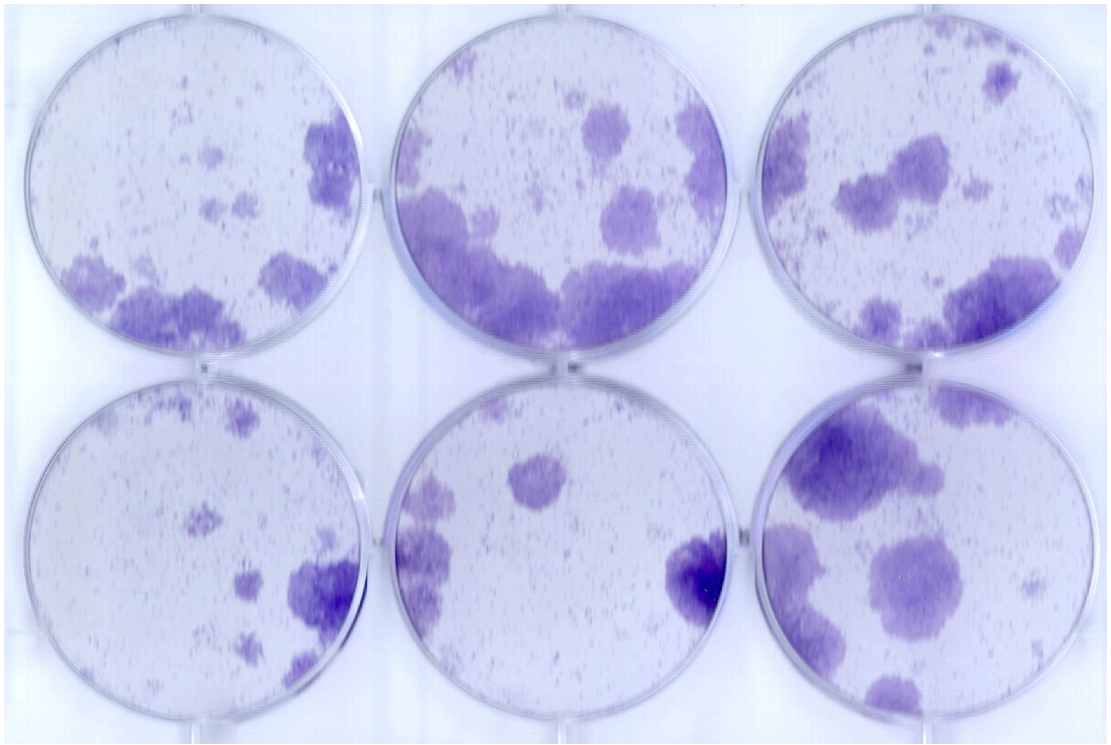
- Seeding density empirically determined
- Ideally want 20+ colonies per well that are still countable

Expectation



PC3 prostate cancer cell

Reality



ED501 Glioblastoma primary cell line

Plotting clonogenic assay

A

Dose	# Cells Plated	# Colonies	Mean/X	% PE	% Survival
0Gy	100	82, 78, 80	80	80	100
2Gy	200	55, 57, 48	53.3	26.6	33.3
4Gy	600	48, 46, 41	45	7.5	9.4
6Gy	1000	8, 12, 15	11.6	1.2	1.5

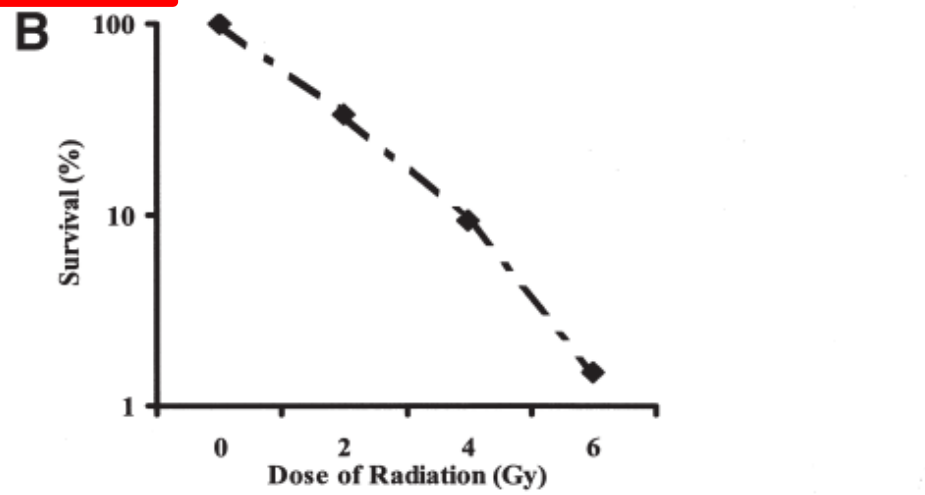


Fig. 2. (A) Setup of dilution sheet used during clonogenic cell survival assays; (B) survival curve plotted using hypothetical numbers derived from dilution sheet

Munshi et al. Methods in Molecular Medicine vol 110.

Plotting clonogenic assay

A

Dose	# Cells Plated	# Colonies	Mean/X	% PE	% Survival
0Gy	100	82, 78, 80	80	80	100
2Gy	200	55, 57, 48	53.3	26.6	33.3
4Gy	600	48, 46, 41	45	7.5	9.4
6Gy	1000	8, 12, 15	11.6	1.2	1.5

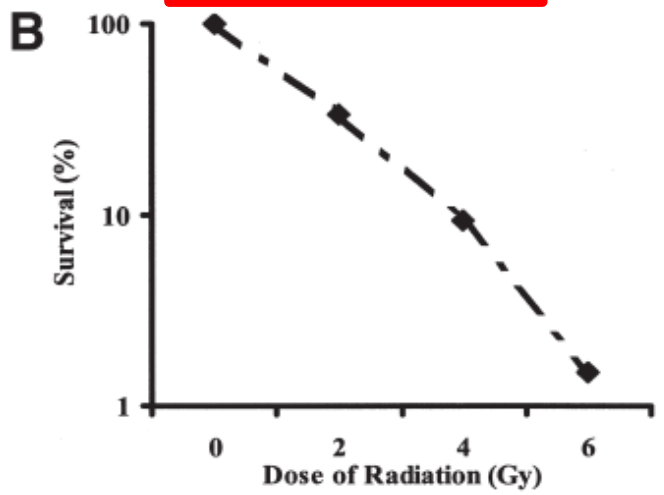


Fig. 2. (A) Setup of dilution sheet used during clonogenic cell survival assays; (B) survival curve plotted using hypothetical numbers derived from dilution sheet

Munshi et al. Methods in Molecular Medicine vol 110.

Plotting clonogenic assay

Colonies/plated

A

Dose	# Cells Plated	# Colonies	Mean/X	% PE	% Survival
0Gy	100	82, 78, 80	80	80	100
2Gy	200	55, 57, 48	53.3	26.6	33.3
4Gy	600	48, 46, 41	45	7.5	9.4
6Gy	1000	8, 12, 15	11.6	1.2	1.5

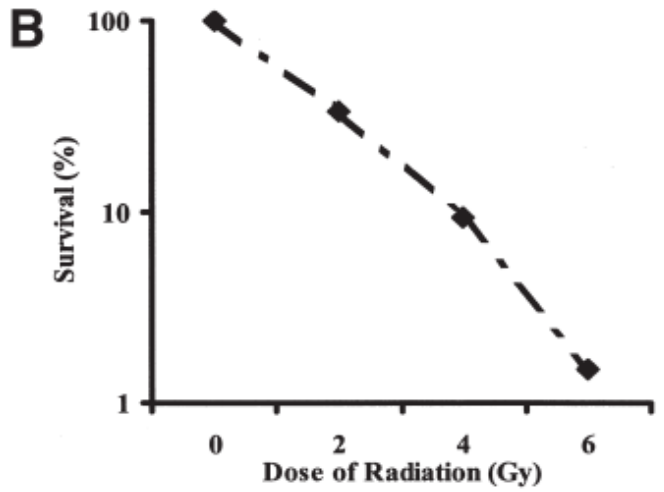


Fig. 2. (A) Setup of dilution sheet used during clonogenic cell survival assays; (B) survival curve plotted using hypothetical numbers derived from dilution sheet

Munshi et al. Methods in Molecular Medicine vol 110.

Plotting clonogenic assay

A

Dose	# Cells Plated	# Colonies	Mean/X	% PE	% Survival
0Gy	100	82, 78, 80	80	80	100
2Gy	200	55, 57, 48	53.3	26.6	33.3
4Gy	600	48, 46, 41	45	7.5	9.4
6Gy	1000	8, 12, 15	11.6	1.2	1.5

Ratio of PE (plating efficiency) for RT dose to control

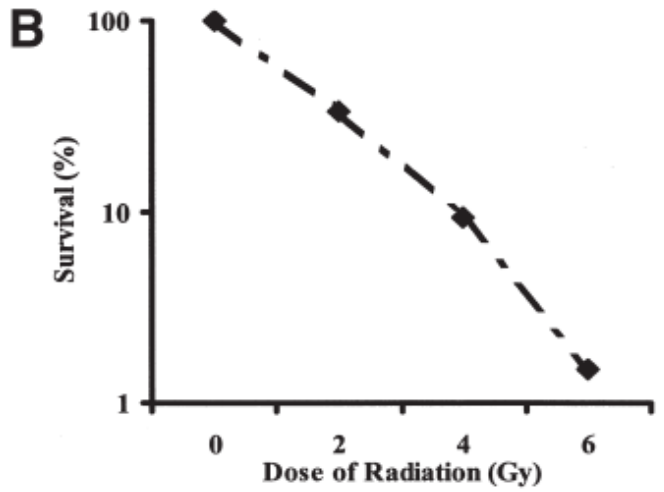
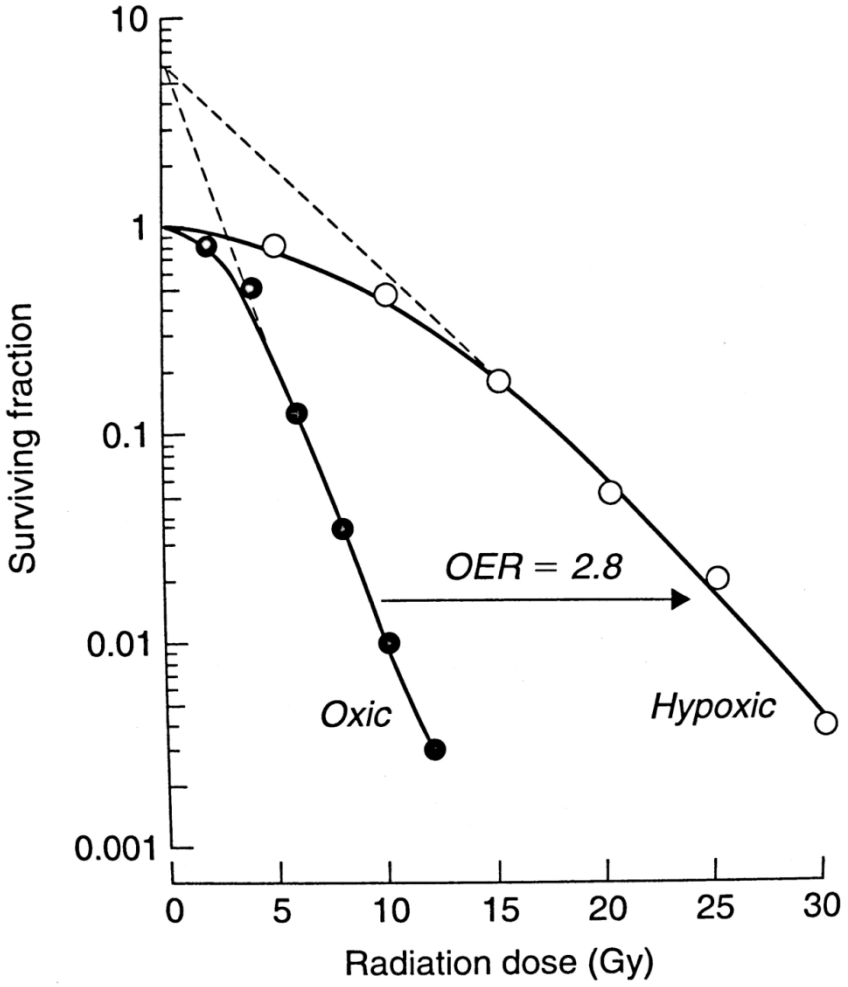
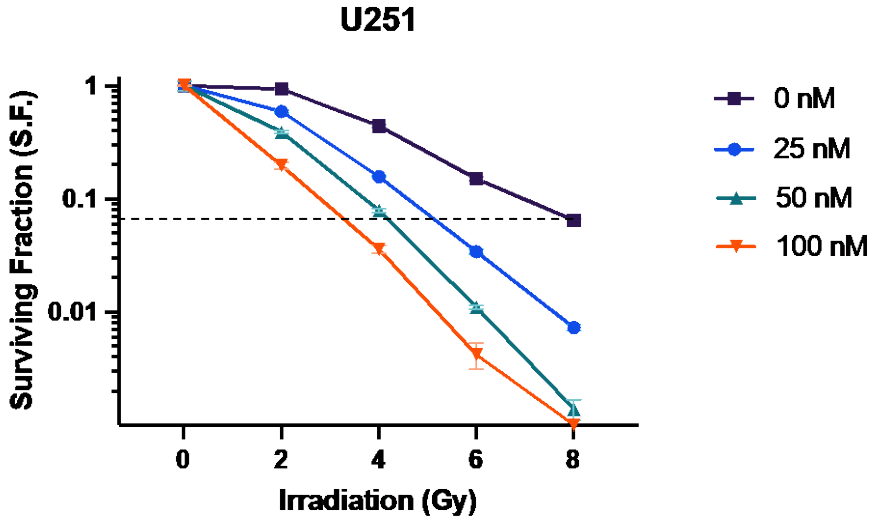


Fig. 2. (A) Setup of dilution sheet used during clonogenic cell survival assays; (B) survival curve plotted using hypothetical numbers derived from dilution sheet

Munshi et al. Methods in Molecular Medicine vol 110.

Example of clonogenic assay to assess differential survival

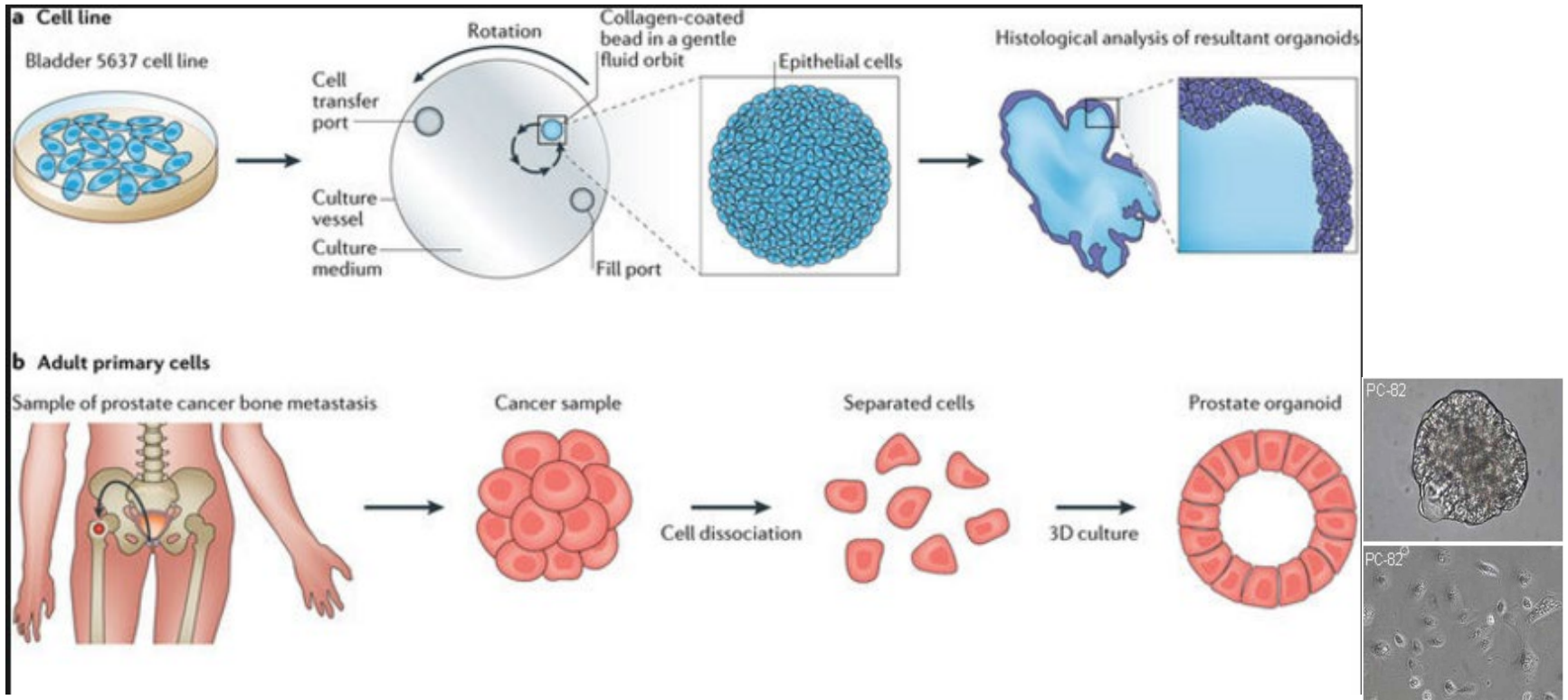




- Dose enhancement Factor (DEF)
  - 25nM – 1.4
  - 50nM – 1.75
  - 100nM – 2.4

Joiner M & van der Kogel A (eds). Basic Clinical Radiobiology. Edward Arnold 2009.

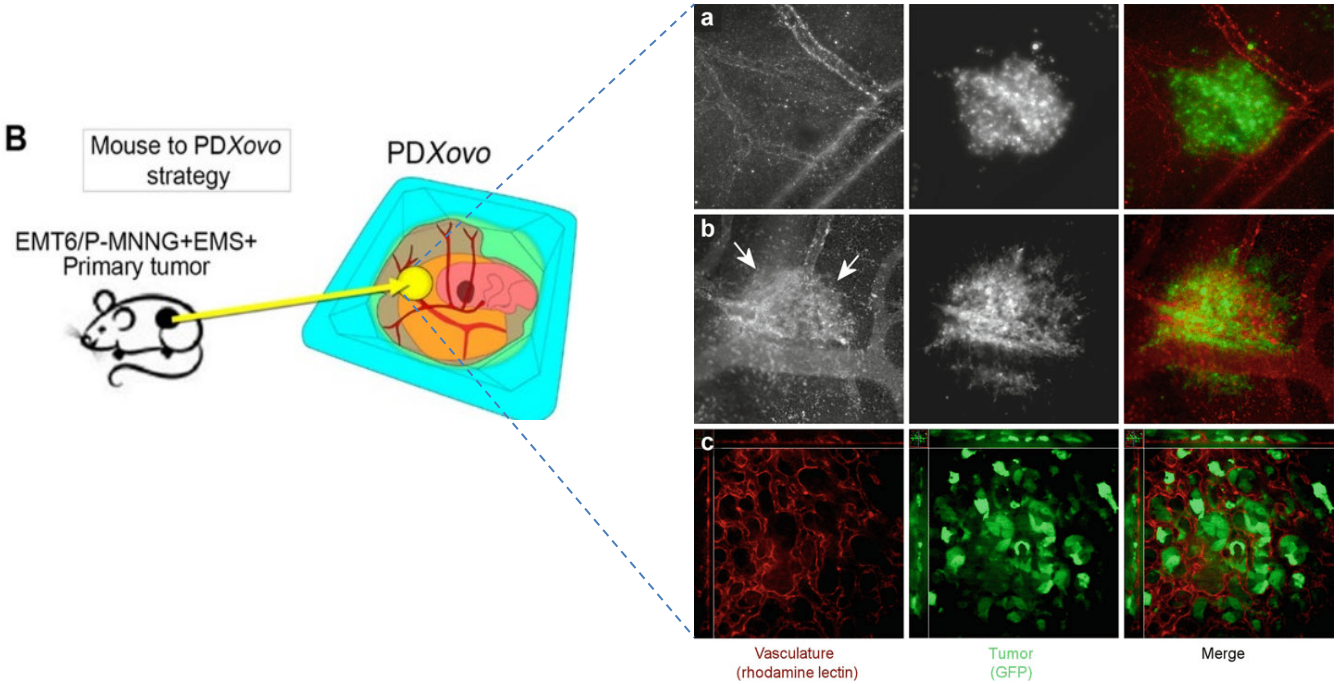
Organoids (3D culture)



- **Can use established cell lines or primary cells from normal or cancerous tissue**
- **Recapitulate 3D *in vivo* architecture , function and genetics of original organ**
- **Will they serve as more predictive 'in vitro' radiation clonogenic assays**

Wang *et al.* Nat Rev Urol 2017
Dutta *et al.* Trends Mol Med 2017

In vivo models: PDX using Chorioallantoic membrane (CAM) model



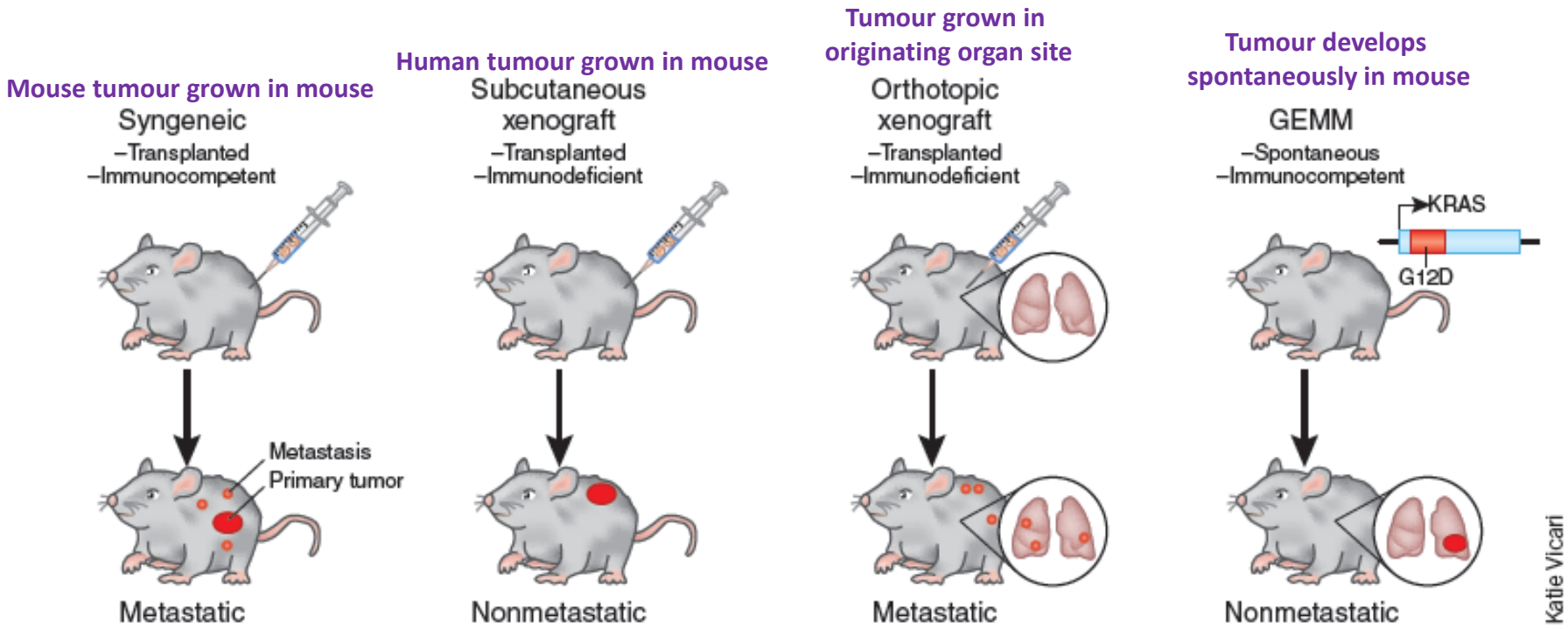
Zijlstra, Andries & Lewis, John. (2012). Visualization and Quantification of De Novo Angiogenesis in Ex Ovo Chicken Embryos.

- **Approximates a mouse for size and blood volume / blood flow**
- **Tumors can be implanted on a flat layer (chorioallantoic membrane)**
- **Can do live intravital imaging**

Wang *et al.* Nat Rev Urol 2017
Dutta *et al.* Trends Mol Med 2017

In vivo Models:
Mouse models to assay tumour growth

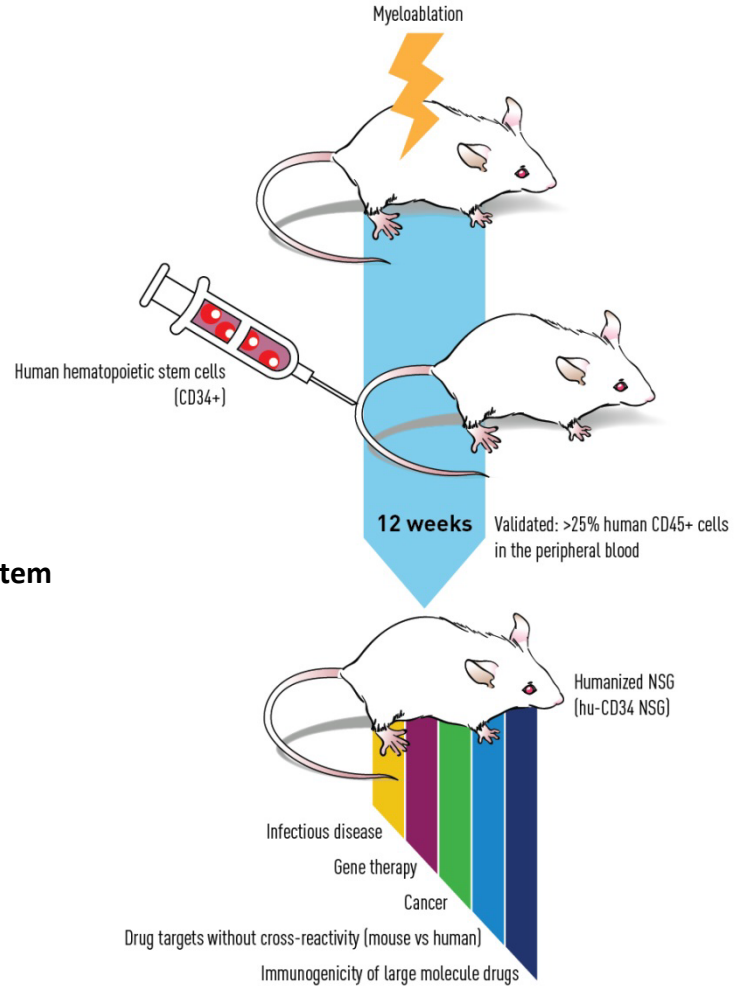
- Immunocompetent or immunodeficient host
- Orthotopic or subcutaneous tumour xenograft



Francia et al Nat Biotech 2010

Humanized PDXs – best of both worlds?

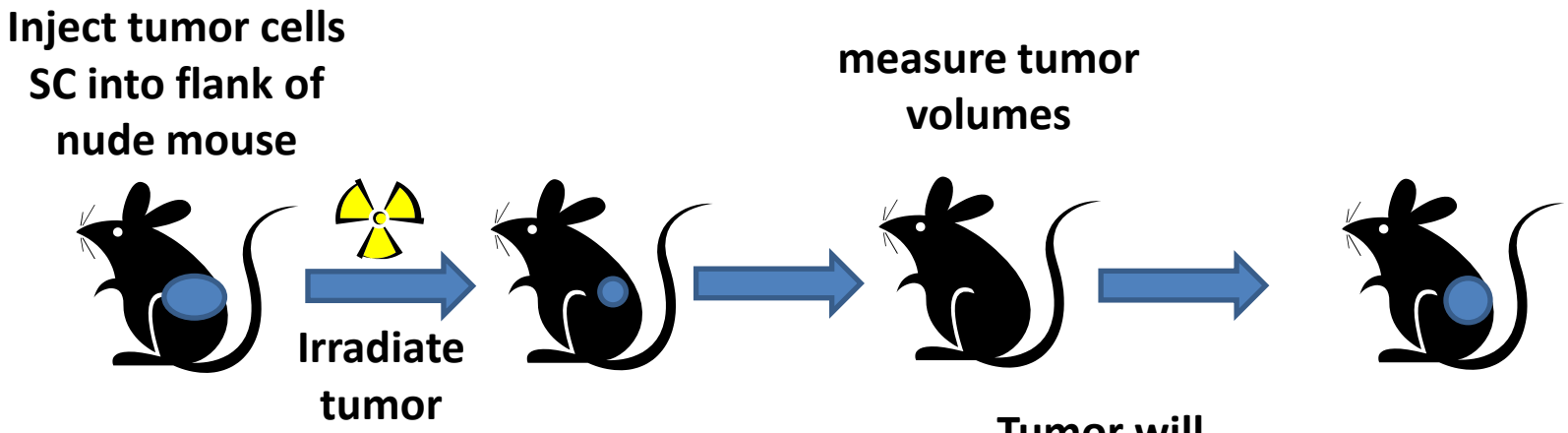
Use patient tumour to generate xenograft in mouse with human immune system

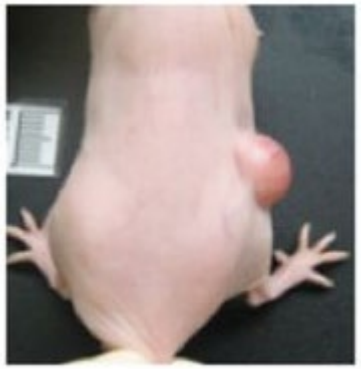


- Allows growth of human tumour within context of human immune system

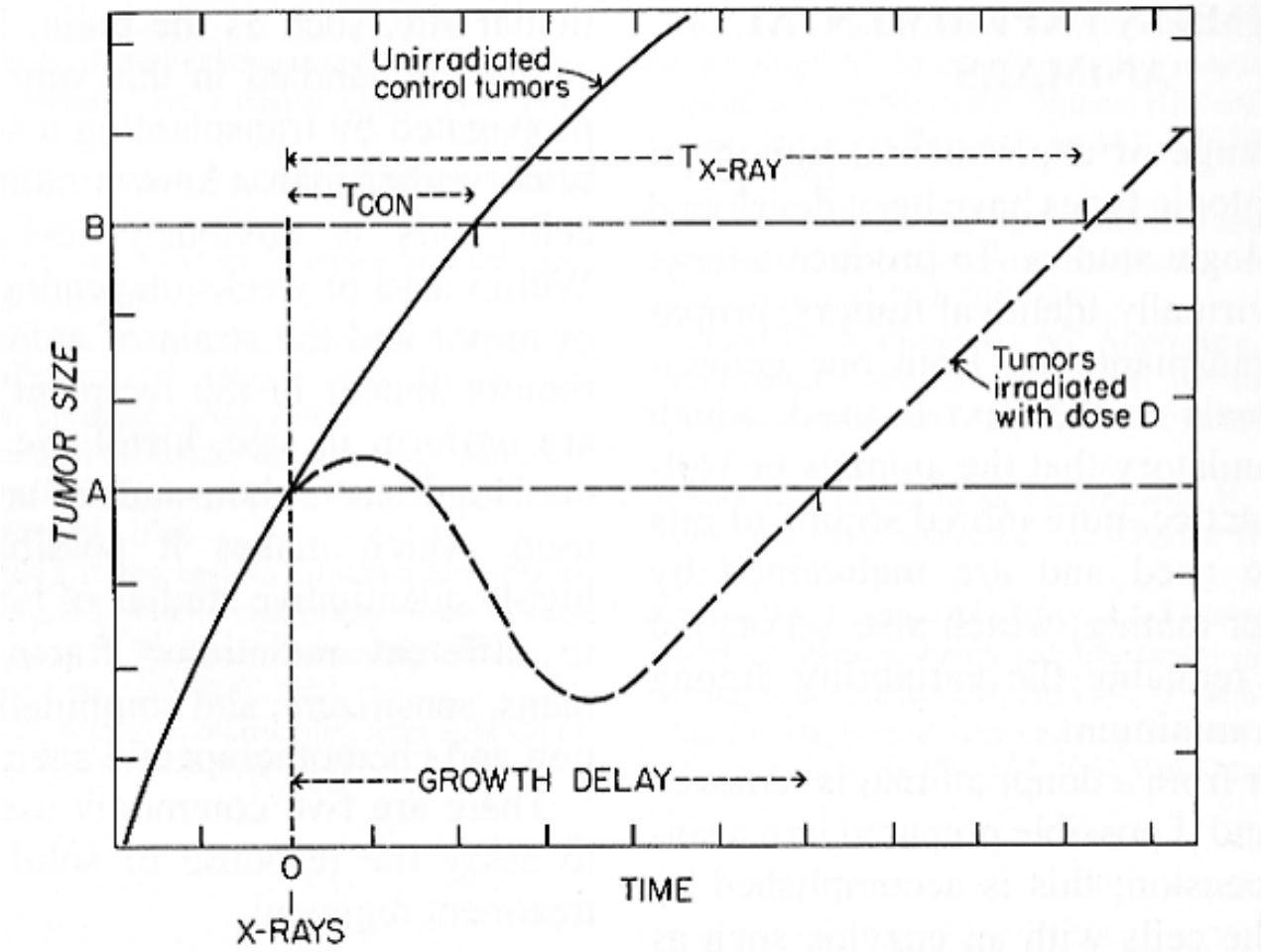
Jax.org

In vivo assay: Tumor growth delay





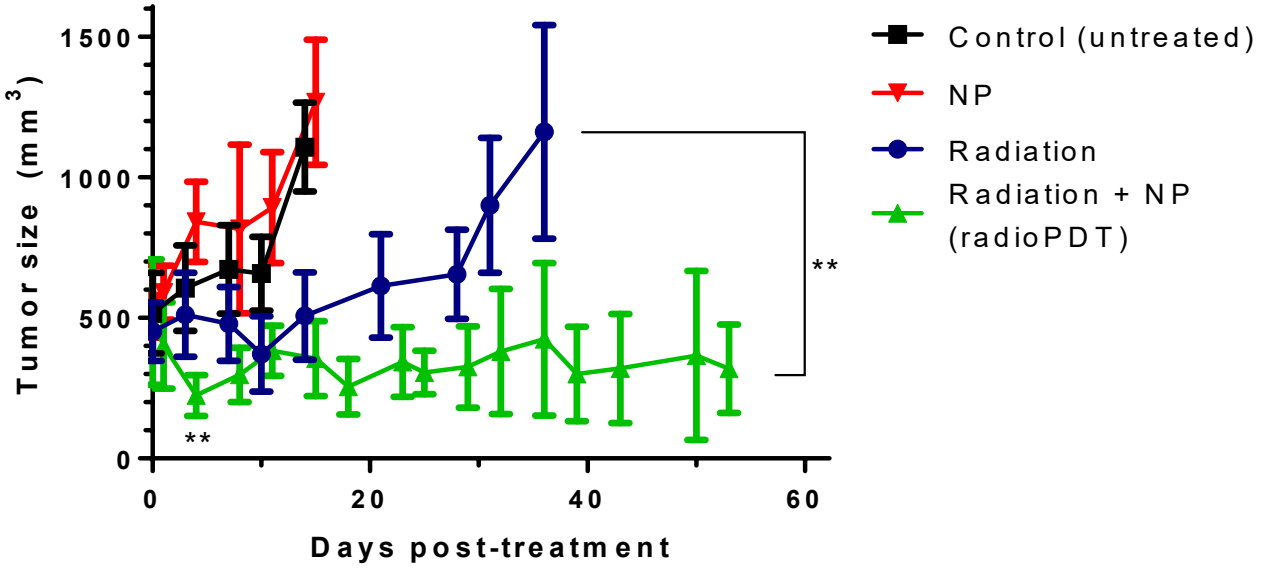
Tumor growth delay



Tumor regresses in volume then regrows after IR

Hall E. Radiobiology for the Radiologist.

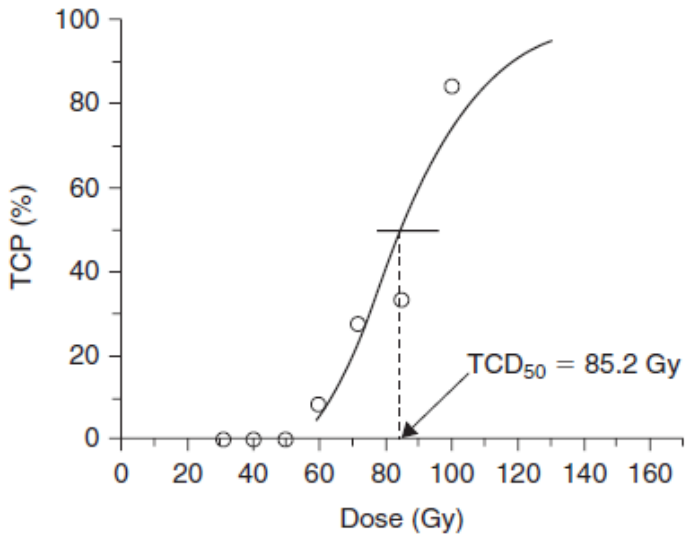
Tumor growth delay



Tumour Control Dose (TCD50)

- Inject mice to form tumour xenografts
- Irradiate mice with increasing doses of radiation
- Determine dose of radiation needed to cure 50% of mice

TCD50



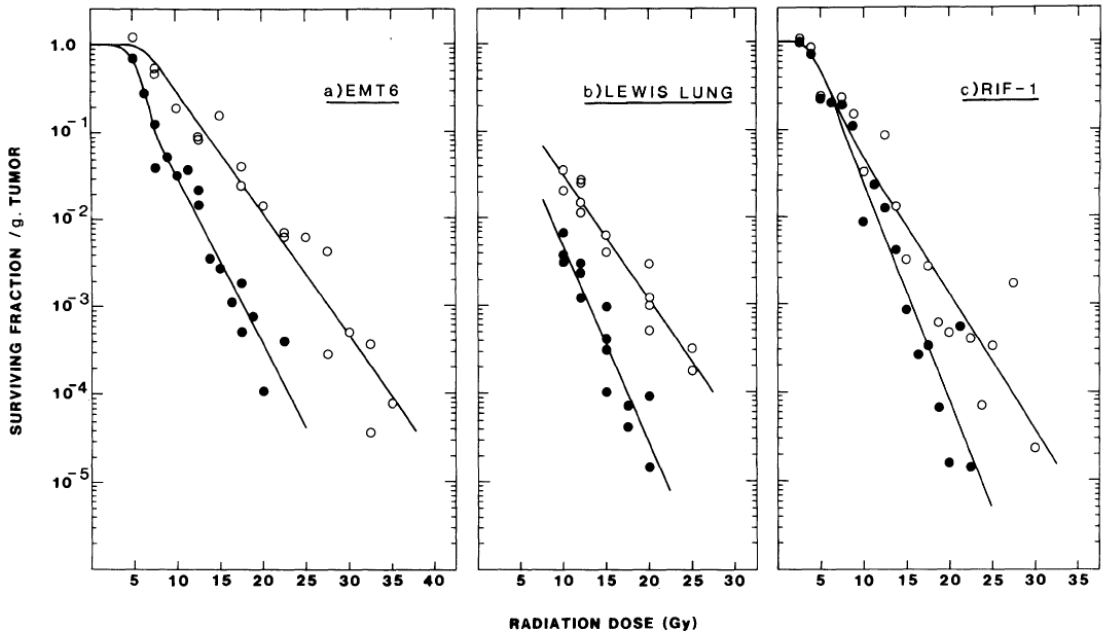


FIG. 4. The effect of nicotinamide (1000 mg/kg) on the X-ray dose-response curve in three different tumor models. Nicotinamide was injected either 90 (EMT6), 60 (Lewis Lung), or 120 min (RIF-1) before irradiation. Tumor survival was measured 24 h after irradiation. Open circles, saline + X rays; closed circles, nicotinamide + X rays. Individual data points from three separate experiments are shown with the lines determined by linear regression analysis.

Horsman et al. *Rad Res* 1987

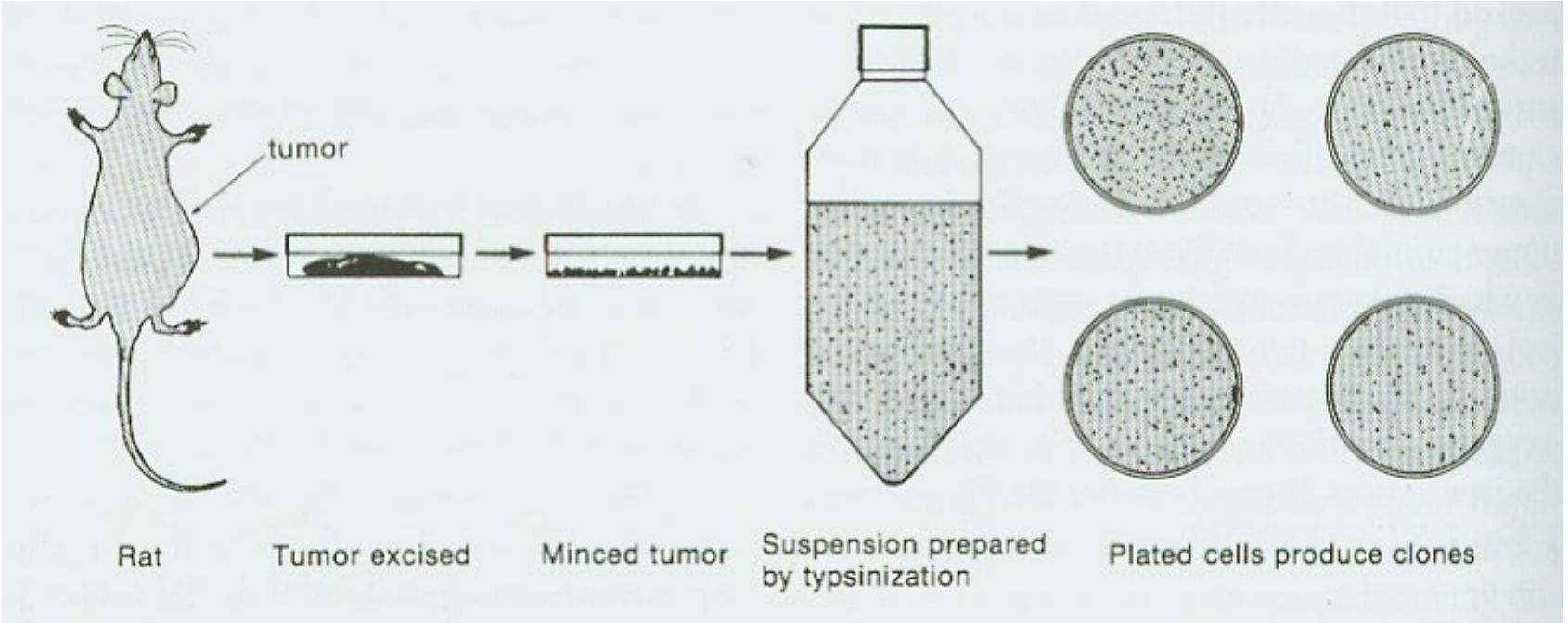
Joiner & van der Kogel. Basic Clinical Radiobiology 4th ed

Alternative: *Ex vivo* clonogenic assay

Irradiation *in situ*

Excise tumor and obtain single cell suspension

Setup clonogenic assay



Hall E. Radiobiology for the Radiologist.

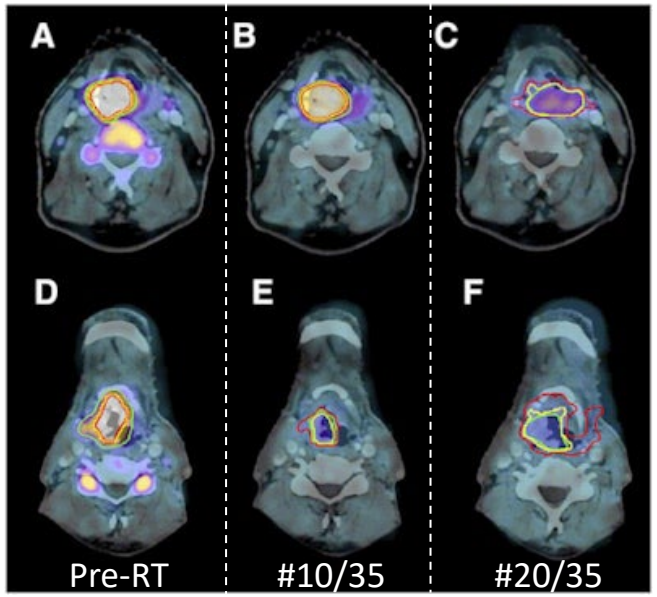
Ex vivo Clonogenic survival assays

- Radiation clonogenic survival assays
  - Adv: quick, cheap, measures clonogenic survival
  - Dis: cannot replicate *tumor microenvironment* (fluctuating hypoxia, IFP, stromal cells, immune system)

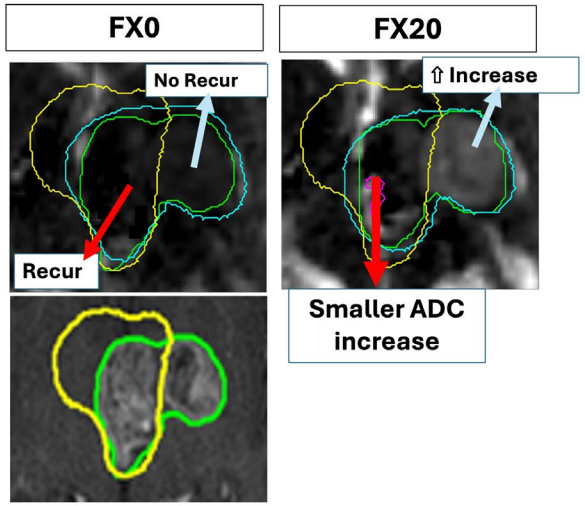
In vivo assays

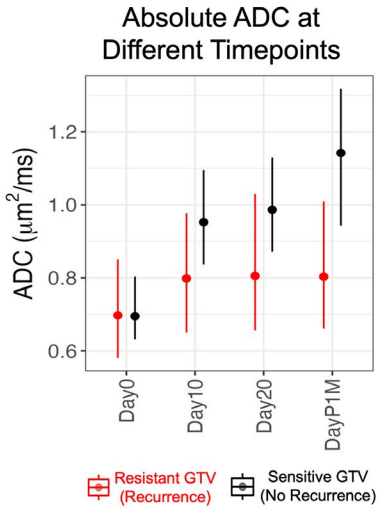
	Tumor growth delay	TCD50	Ex-vivo clonogenic assay
Advantages	cheaper and easier than TCD50	measures clonogenic survival; considered most important assay for curative radiation effects	measures clonogenic survival, quicker than other two assays
Disadvantages	doesn't measure clonogenic survival (kill of clonogenic and non-clonogenic cells)	very expensive, time consuming	does not account for ongoing effects of tumor microenvironment

In clinic – evolving surrogates



Hoeben et al. JNM 2013





Palhares et al. unpublished

^{18}F-FLT PET

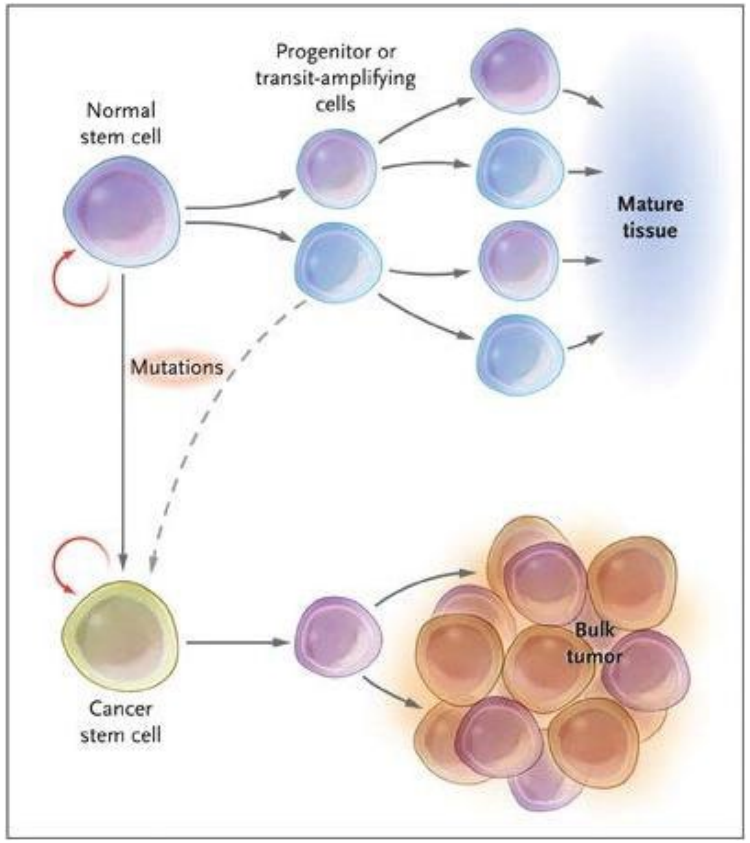
- Marker of proliferative index
- Early response indicator to RT

MRI tissue diffusion (ADC) as marker of cellularity

- Persistently low ADC (i.e. still high celluarity) in RT course predicts for more radioresistance

Beyond clonogenics: Cancer Stem Cells (CSCs)

- Possess **self-renewal** property like normal stem cells
- **Slow proliferation** rate unlike 'bulk' non-CSCs
- May reside in a microenvironmental niche



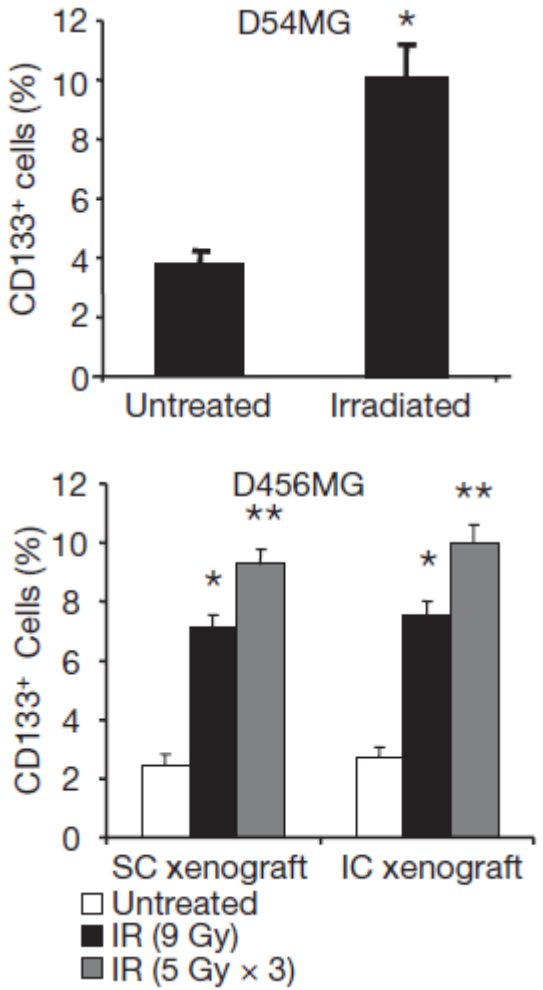
Jordan et. al. NEJM 2006

Cancer Stem Cells (CSCs)

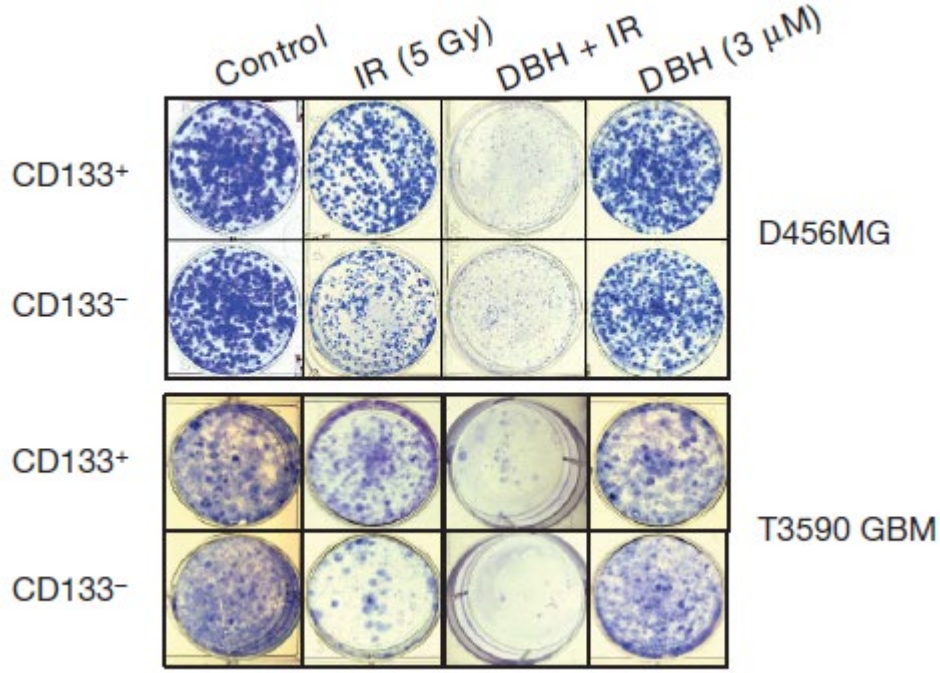
- Identified on basis of specific cell surface markers (e.g., CD133, CD44) or functional properties (ALDH+, drug efflux)
- Have high intrinsic tumorigenic potential
- **Radio/Chemo-resistant**
  - **May involve increased DNA repair, activation of pro-survival pathways, protection from free radicals**

CSCs are enriched by radiation treatment

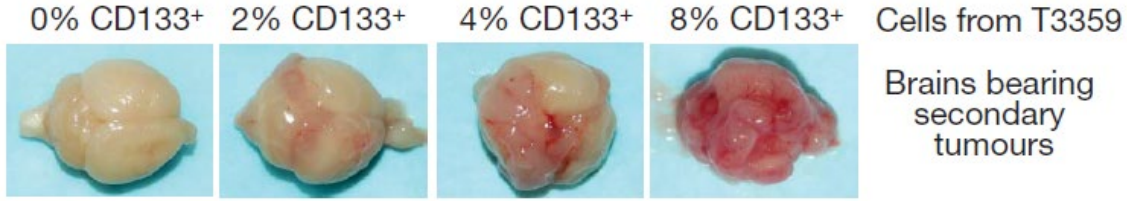
Radiation *in vitro* and *in vivo* enriches for CD133+



CD133+ cells are more radiation resistant



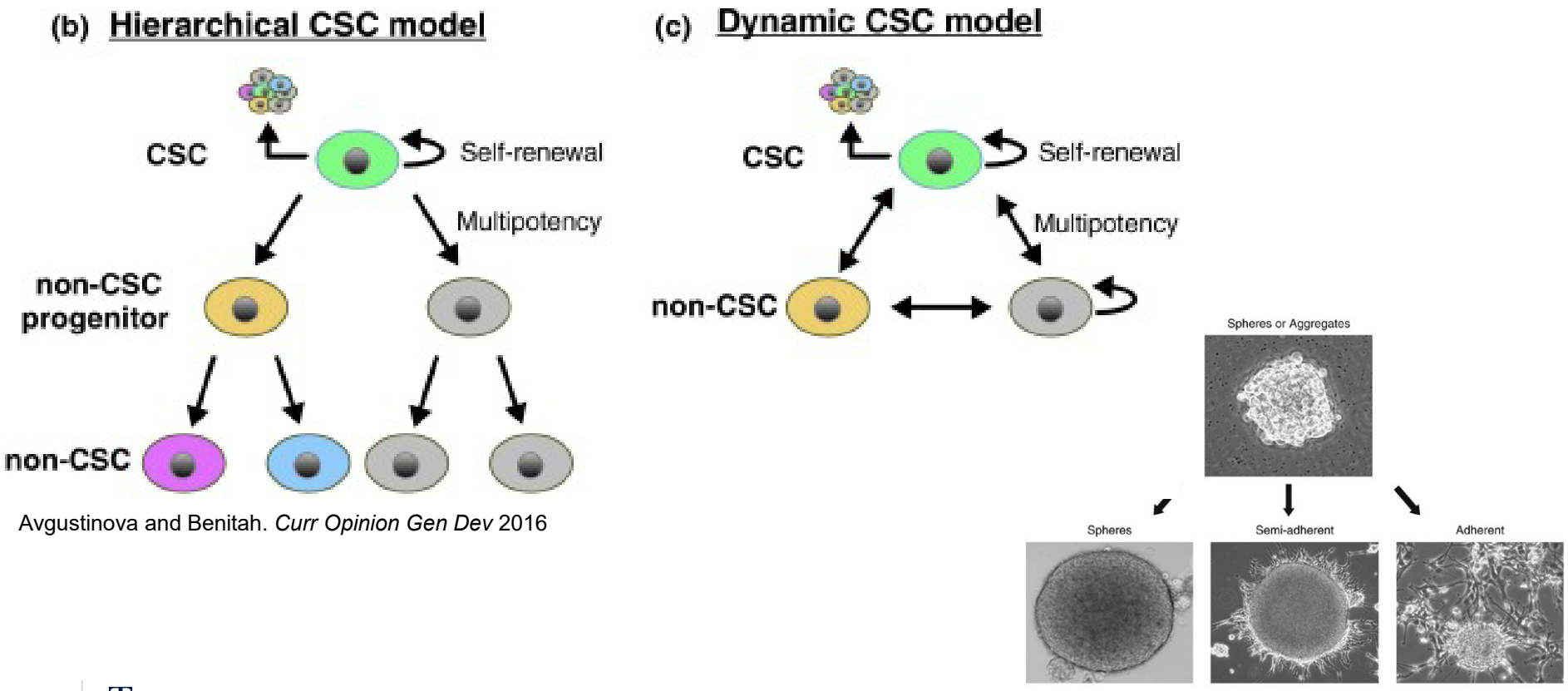
CD133+ cells form more aggressive, vascular tumours



Bao *et al.*, *Nature* 2006

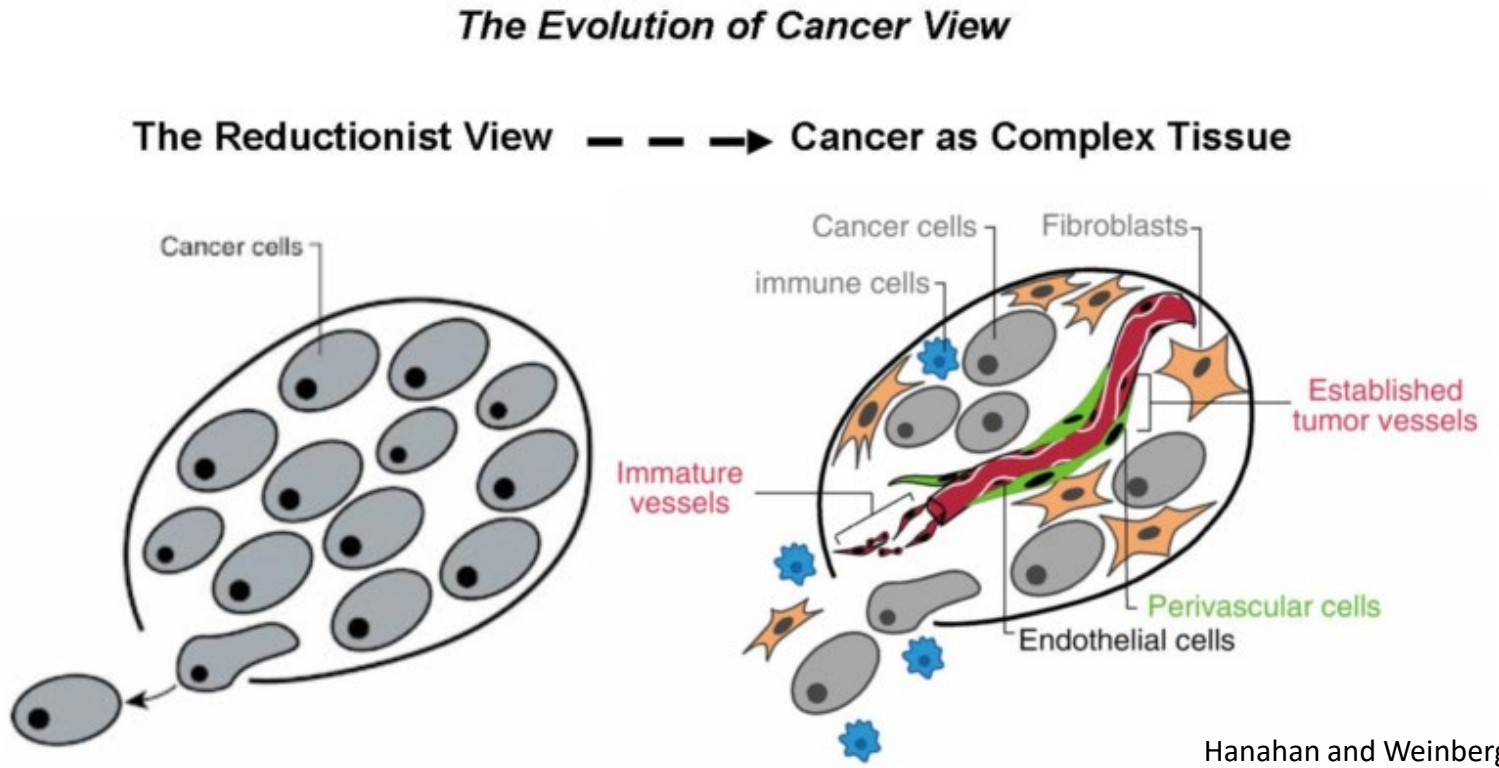
Plasticity of CSCs?

- CSCs may display plasticity (i.e., non-CSCs to CSCs)
- Importance of eliminating both CSCs and non-CSCs?



Avgustinova and Benitah. *Curr Opinion Gen Dev* 2016

What is the tumour microenvironment?

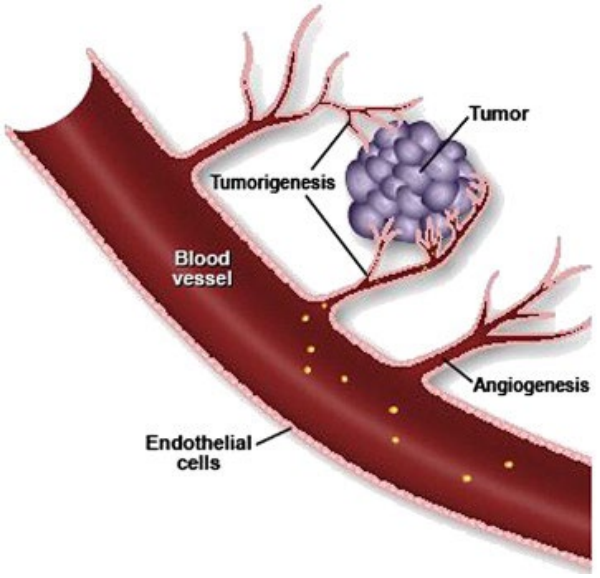


Hanahan and Weinberg. Cell 2000

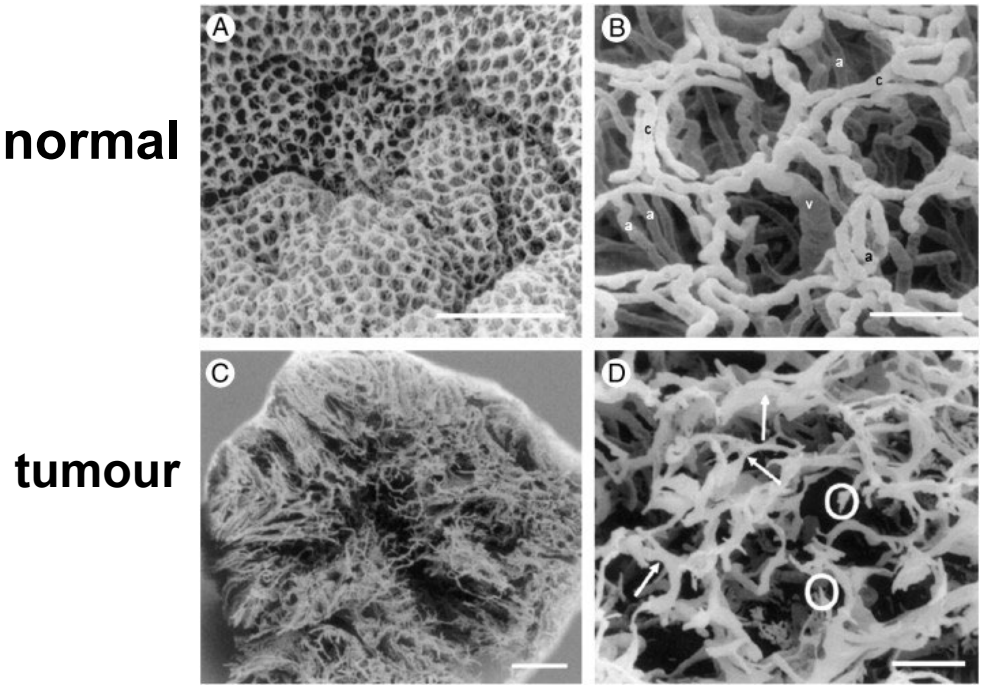
- Main components of TME:
  - Vasculature
  - Immune cells
  - Interstitial tissue (ECM, fibroblasts)
- Effects tumor growth through:
  - Secreted factors
  - Direct cell-cell contact

TME components: Vasculature

- Vasculature is a major functional component in microenvironment
- Required for continued tumour growth and spread



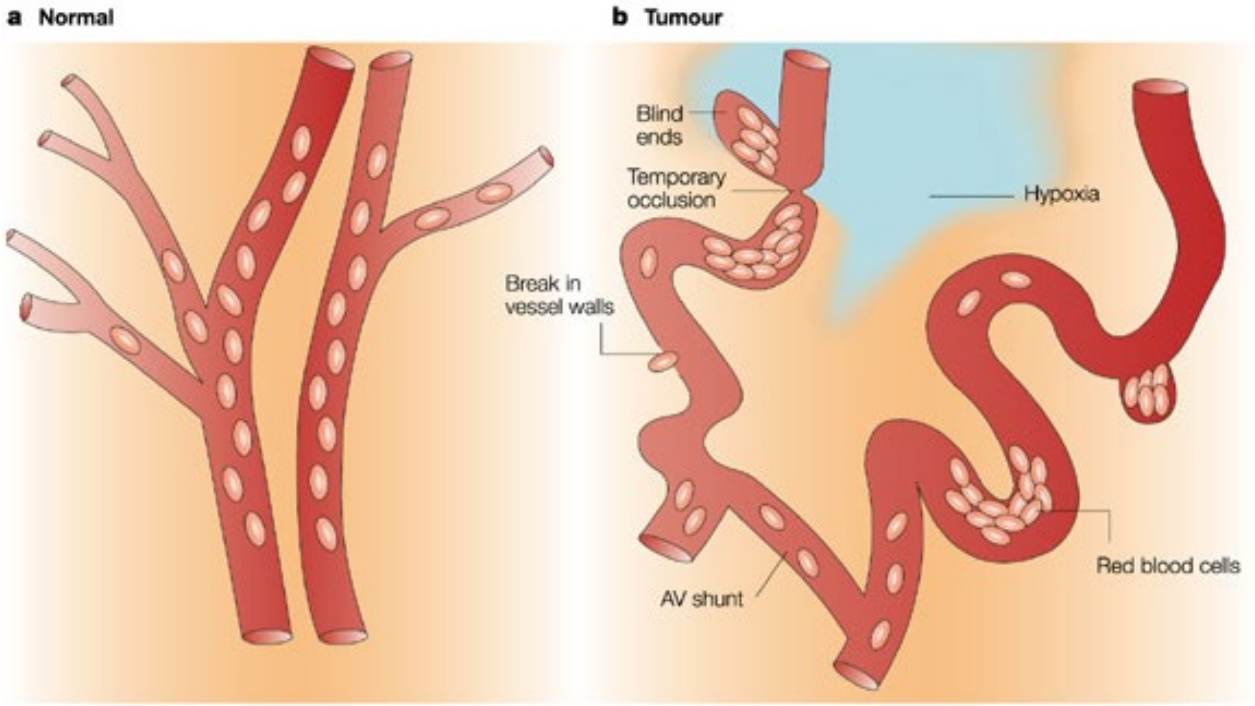
Bhattacharya et al, Adv Drug Deliv Rev 2018



Narang and Varia, Adv Drug Deliv Rev 2011

Vasculature

- Tumour vasculature is abnormal:
  - Leaky, torturous, blind ends, shunts
  - Resulting in **<u>poor tumour perfusion and hypoxia</u>**



RT's effect on vasculature: #1 decreasing vascular density

As IR dose ↑, vascular density ↓

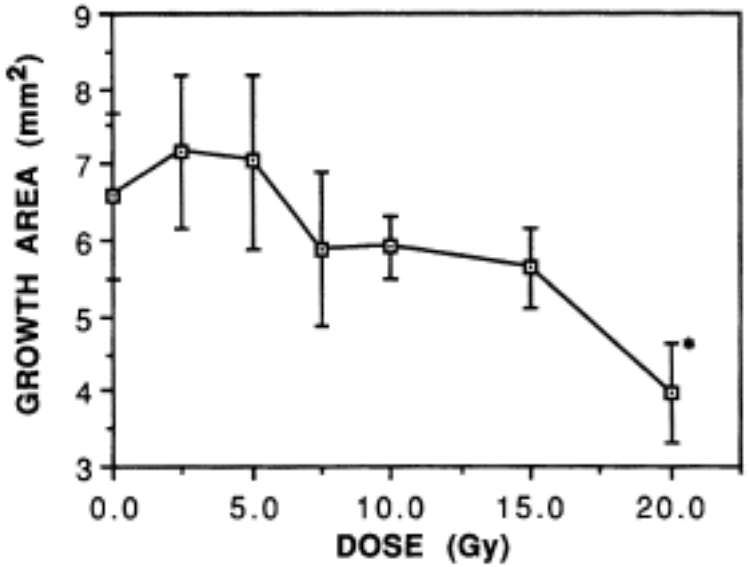


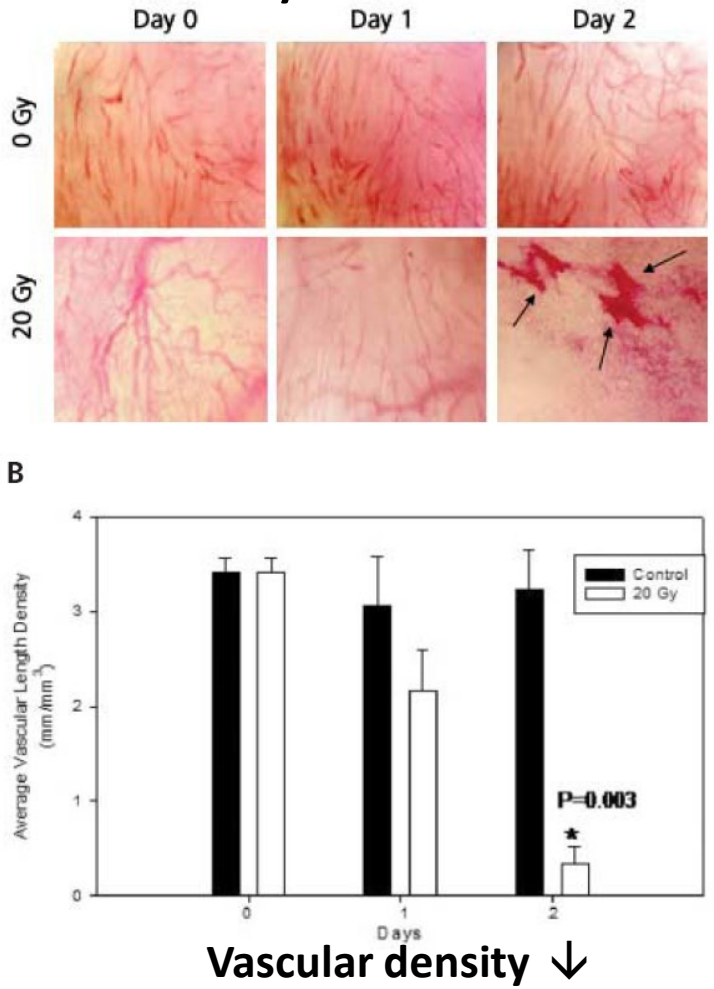
FIG. 6. Radiation dose–response relationship. The total growth area of the angiogenesis discs was measured using a digital video image analysis technique (see text for details). X irradiation was given on Day 11, and the discs were extracted on Day 20 after implantation. Each point represents the mean ± SE of four to six animals. The extent of total growth area achieved in unirradiated discs on Day 11 was 4.4 mm².

Prionas et al., *Radiation Res* 1990.

RT's effect on vasculature: #1 decreasing vascular density

As IR dose ↑, vascular density ↓

- Appears to be a dose-dependent effect on vasculature function
- At **lower doses** (e.g., < 8 Gy) – no significant effect
- At **higher doses (**e.g., **stereotactic RT)**, vascular ablation is believed to occur which may indirectly result in death of tumor cells

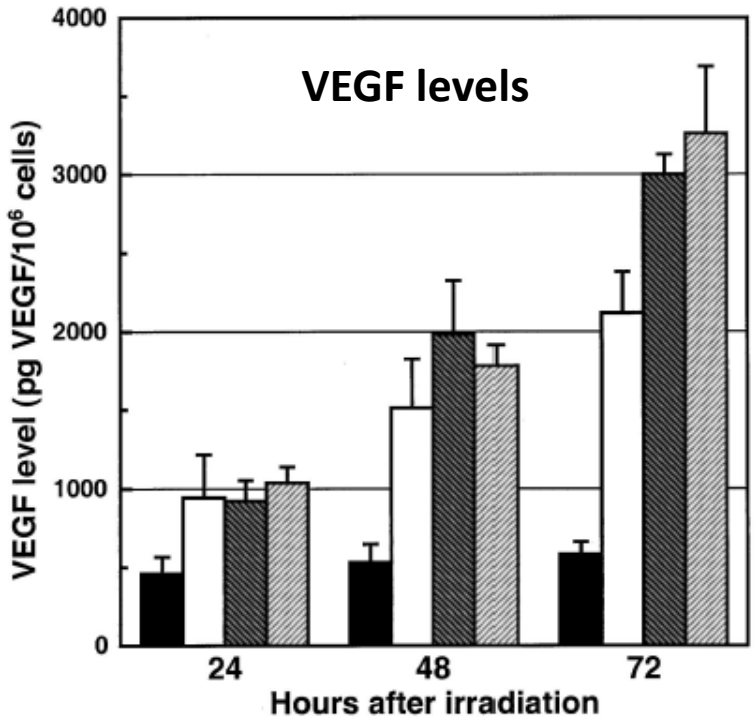


Vascular density ↓

Kim et al., *J US Med* 2006.

RT increases vascularity through angiogenic factors

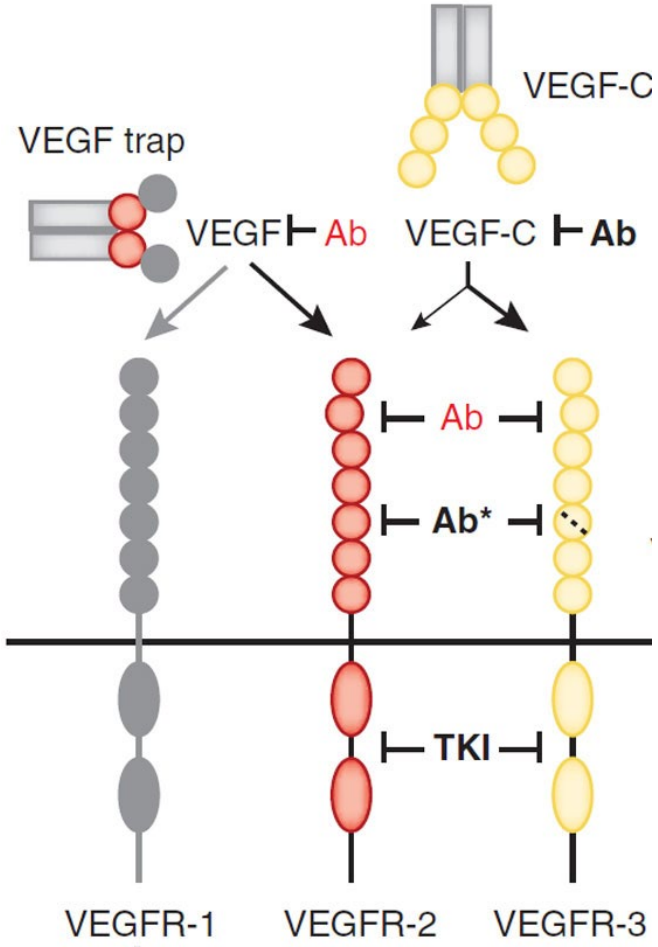
- promotes tumour secretion of **VEGF**



10, or 20 Gy. Conditioned medium was collected every 24 h, and VEGF levels were normalized to cell number. A dose-dependent increase in VEGF secretion was observed for all doses of IR ($P < 0.05$). ■, 0 cGy; □, 500 cGy; ▧, 1000 cGy; ▨, 2000 cGy. Data are presented as means; *bars*, SE. *C*, VEGF expression in human tumor cell

Gorski et al. *Cancer Res* 1999

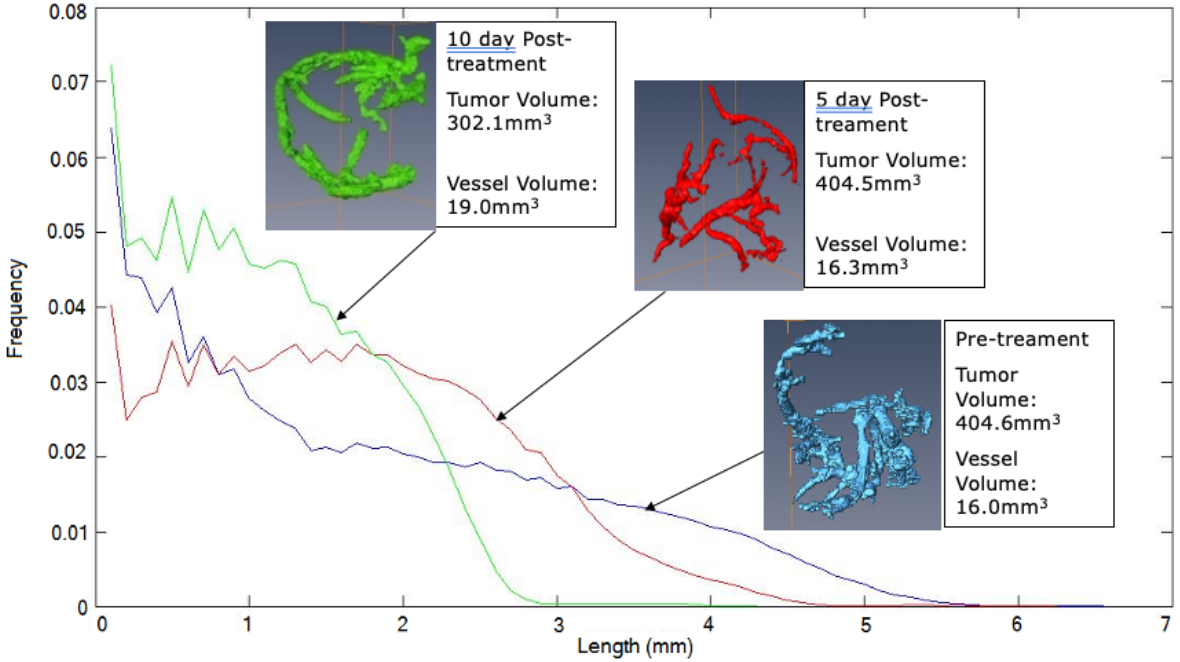
Modulationg Tumor vasculature: Inhibiting the VEGF pathway



Alitalo *Nat Med* 2011.

- **Antibodies**: bevacizumab
- **Tyrosine-kinase inhibitors:** Lenvatinib (sorafenib, sunitinib, axitinib, multiple others)

Real world effects: decreases vascular neoangiogensis, normalizes vasculature, decreases vessel “leakiness”

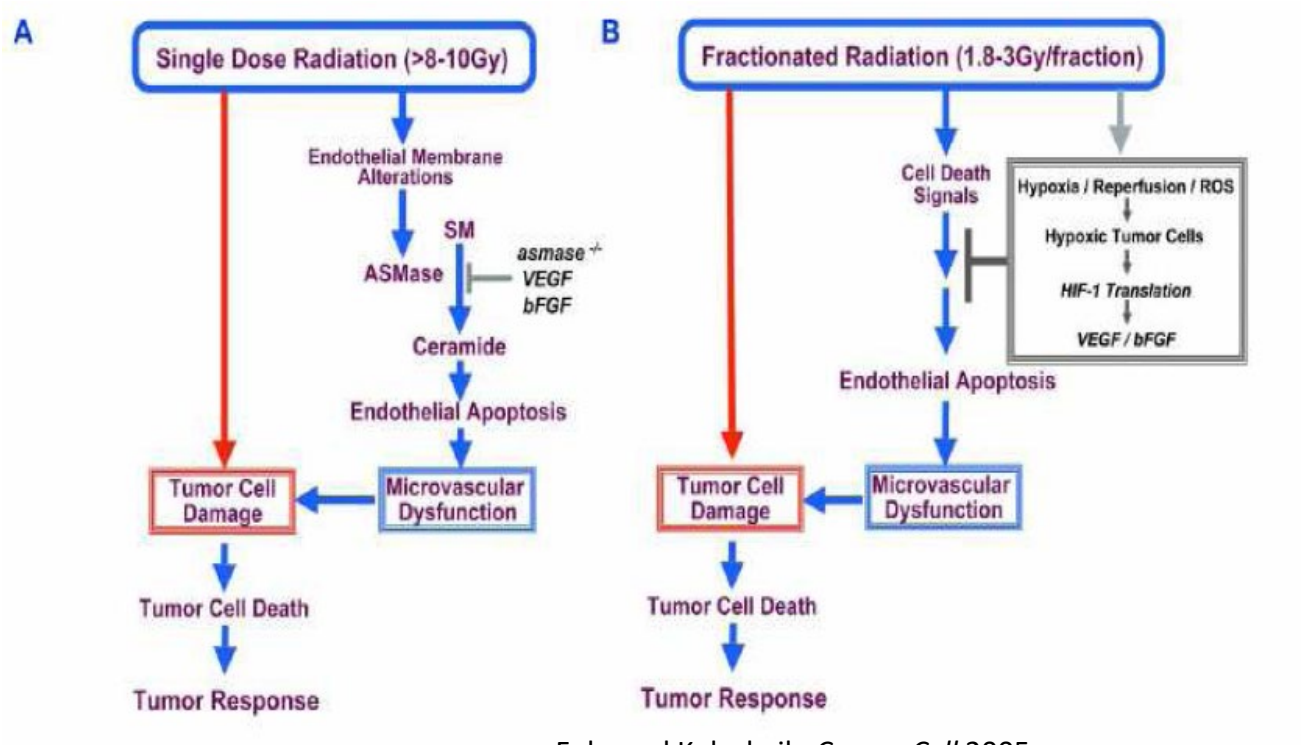


Bevacizumab injected into MDA-MB-231 implanted orthotopic mouse model

Ceramide-mediated endothelial apoptosis – dose dependent induction?

Stereotactic Radiation
-Highly focused, high dose per fraction
-For tumour ablation

Conventional Radiation
-Standard fields and dose per fraction
-For tumour cell death



Fuks and Kolesknik, *Cancer Cell* 2005

RT's effect on vasculature:
#2 endothelial damage inducing ceramide signaling

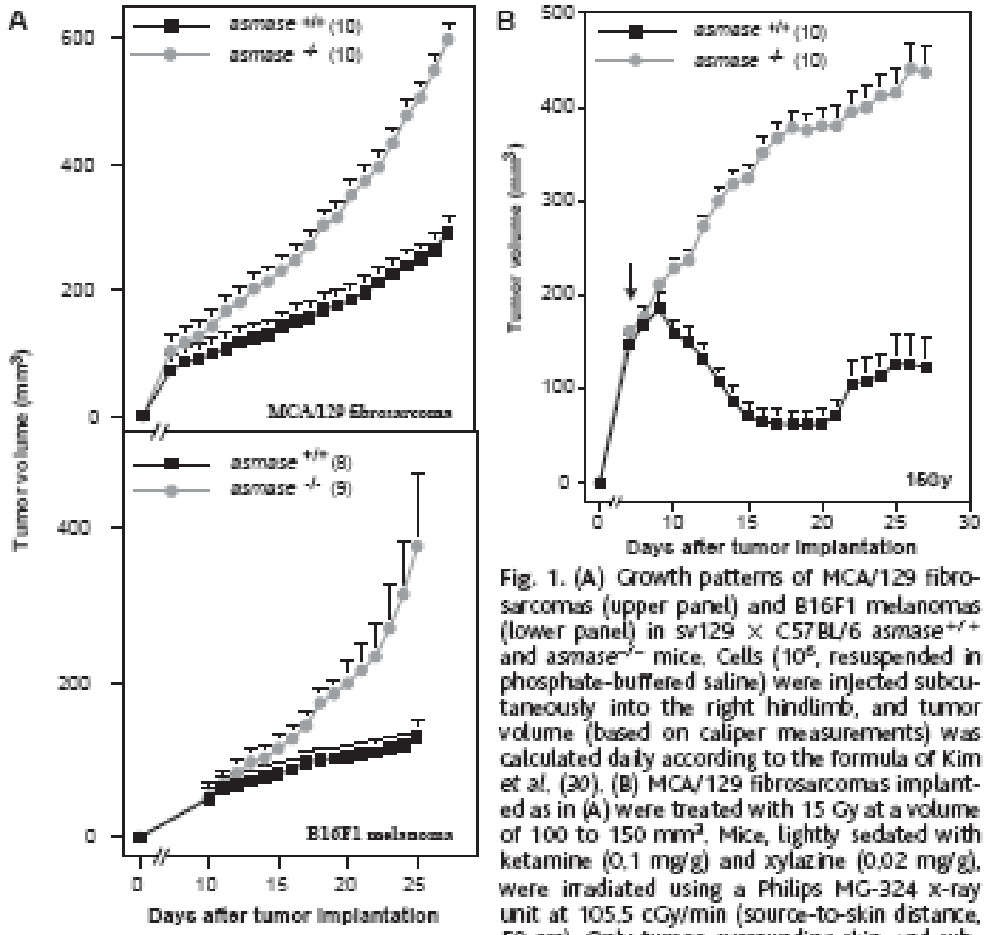
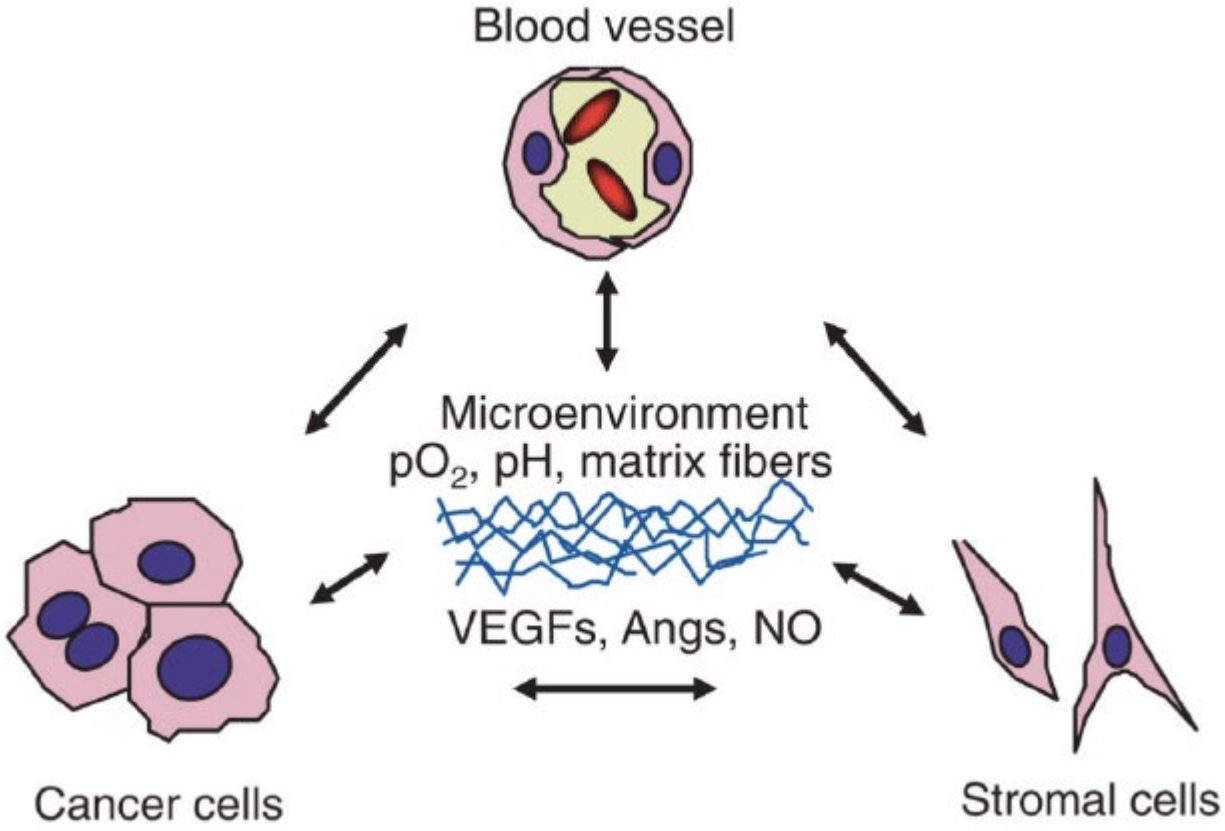


Fig. 1. (A) Growth patterns of MCA/129 fibrosarcomas (upper panel) and B16F1 melanomas (lower panel) in sv129 × C57BL/6 *asmase*$^{+/+}$ and *asmase*$^{-/-}$ mice. Cells (10^6, resuspended in phosphate-buffered saline) were injected subcutaneously into the right hindlimb, and tumor volume (based on caliper measurements) was calculated daily according to the formula of Kim *et al.* (30). (B) MCA/129 fibrosarcomas implanted as in (A) were treated with 15 Gy at a volume of 100 to 150 mm³. Mice, lightly sedated with ketamine (0.1 mg/g) and xylazine (0.02 mg/g), were irradiated using a Philips MG-324 x-ray unit at 105.5 cGy/min (source-to-skin distance, 50 cm). Only tumor, surrounding skin, and subcutaneous tissues were exposed; the rest of the mouse was shielded with a specialized lead jig. Values are means ± SEM. Numbers of mice are in parentheses. Arrow indicates day of irradiation.

Garcia-Barros et al. Science 2003

Tumors have marked resistance to single high dose radiation (15 Gy) when endothelial apoptosis is inhibited *This remains an area of controversy*

Communication within the tumour microenvironment – cell interactions and secreted factors



Fukumura *et al*. Microcirculation 2010

Summary – *in vitro/ in vivo growth* assay

- Tumour volume growth is a balance of doubling time vs cell loss – both are highly variable and have a large influence
- Clonogenic assays attempt to identify fraction of cells capable of creating new clones to predict tumour growth
- Different radiation response assays exist
  - *In vitro* surviving fraction (clonogenic)
  - *In vivo* TCD50 (clonogenic) – gold standard for curative radiation
  - Tumour growth delay (clonogenic and non-clonogenic effects)
  - *Ex vivo* surviving fraction (clonogenic)
  - Clinical early radio-response biomarkers ex:FLT-PET and MRI-ADC (clonogenic and non-clonogenic)
- Important to consider tumour model used
  - Immune status, orthotopic vs subcutaneous

Summary – impact of nonclonogens (CSCs)

- Cancer Stem cells (CSCs) may contribute to radiation resistance
  - Promotes more aggressive cancer phenotype
  - More radioresistant
  - Can exhibit plasticity (differentiate and de-differentiate into stem cell state)

Summary – TME effects (vasculature)

- RT can affect vasculature
  - Dose-dependent decrease in vascular density
  - At high doses - ceramide-mediated endothelial apoptosis

- RT induces tumour secretion of VEGF, promoting angiogenesis – a potential target

We’d love your feedback!

Lecture Evaluation

Program Evaluation