Bioprinting personalized spare parts
How far away are we?

Technical Journal Club
12.02.2019

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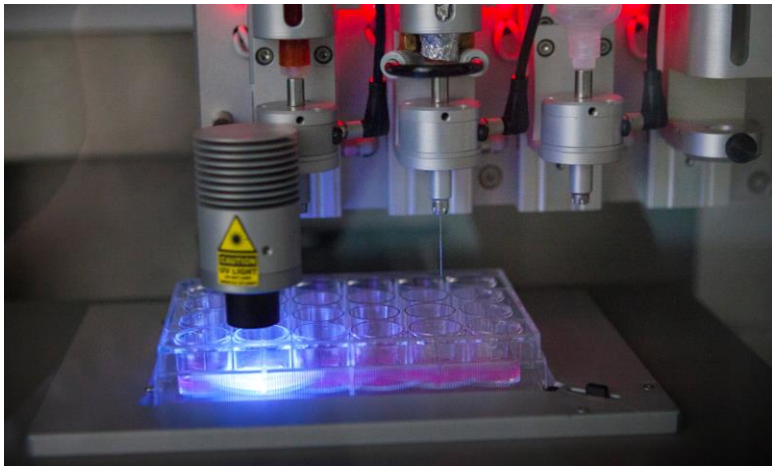
Prospective usage of bioprinting



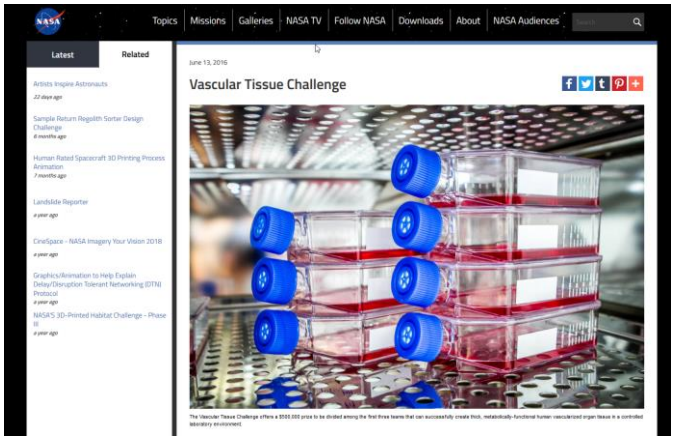
A RegenHU 3D bioprinter at work at Zurich University of Applied Sciences. Photograph: BSIP/UIG via Getty Images

- Teaching and surgical planning
- Personalized surgical guides
- Increasing surgical precision
- In vitro/personalized drug tolerance testing
- Pathologic tissue printing for drug efficacy testing
- Implants, transplants, prostheses, reconstructive surgery
- Printing autologous organs – no need to wait for donors and immunosuppressives
- Face transplants – overcome identity issue by creating a graft similar to original face
- Reduce and refine animal studies

How far away are we?

- Bioprinted skin could be 5 years away
- Implants in cartilage field could be seen in 10 years
- Bioprinting a heart may be possible within less than 10 years
  - Heart is less complicated – no complex biochemistry involved
  - Bioprinting would include a biodegradeable scaffold where cells could be seeded on
- Problem with bigger tissue pieces are blood vessels
  - NASA offers a $500.000 prize for the first researchers printing human tissue with a working blood system surviving 30 days in vitro

Erik Gatenholm and Héctor Martínez , co-founders of Cellink

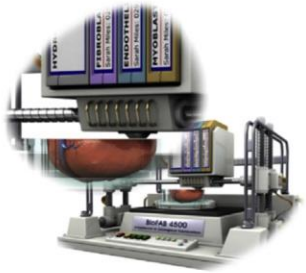


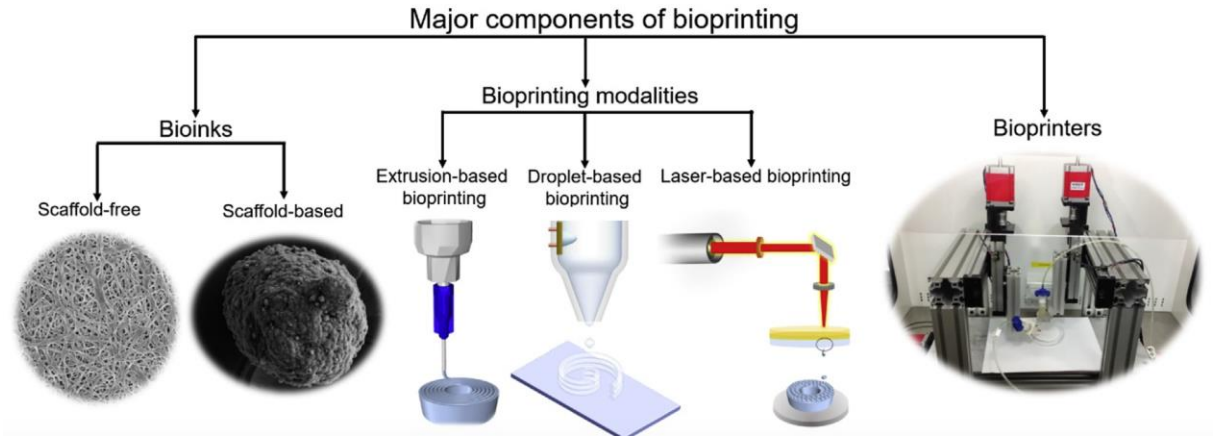
https://www.theguardian.com/technology/2017/jul/30/will-3d-printing-solve-the-organ-transplant-shortage#img-3

How does it work?

- 3 pillars of tissue engineering
  - Cells
  - Scaffold
  - Signals (growth factors)

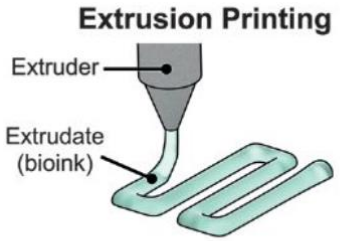
- Major components of bioprinting
  - Bioink
  - Printing modalities
  - Bioprinters

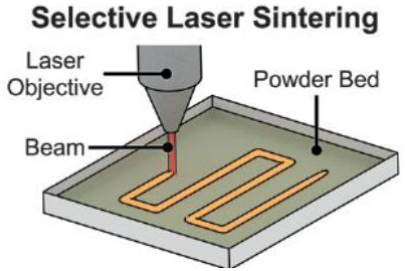


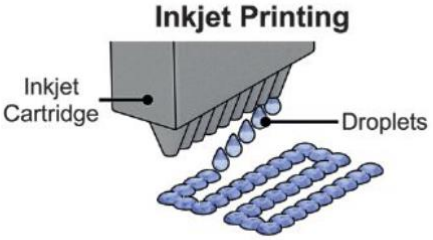


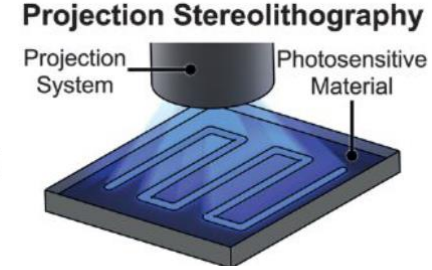
Bioprinting modalities

- **Extrusion Printing:** Thread like deposition using liquid-like materials solidifying in situ
- **Laser sintering:** bed of powder is melted by a laser and a new layer of powder is applied atop the nascent part etc. – high temperatures are not cell compatible
- **Inkjet Printing:** 2D inkjet cartridges with biomaterials and living cells
- **Stereolithography:** photocurable resins and patterned light to solidify material layer by layer
- Photocurable hydrogels and biocompatible photopolymerization enables involving living cells directly in the process

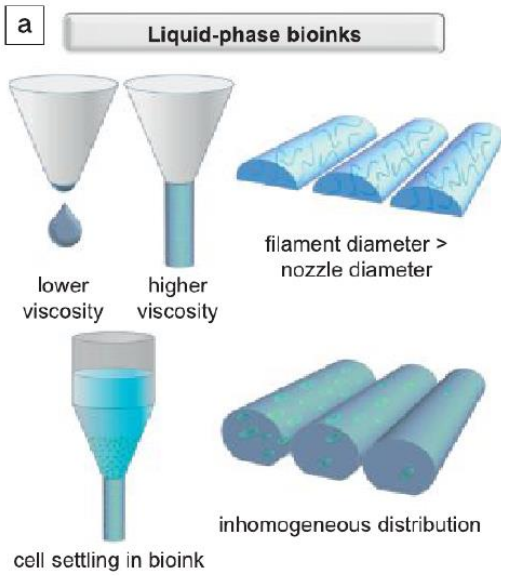


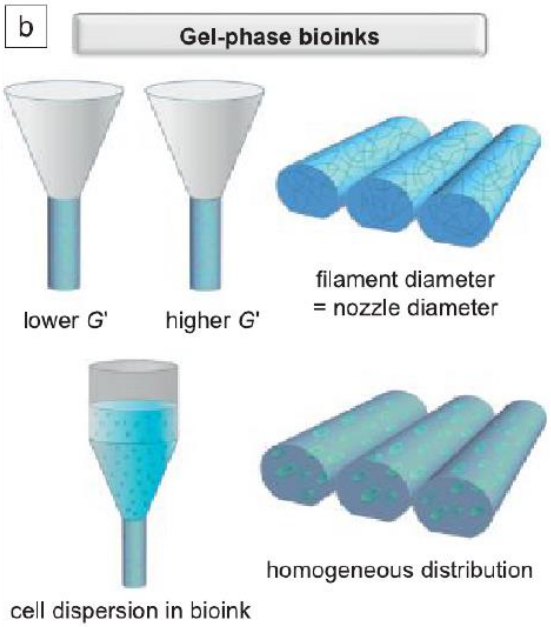






Bioinks for extrusion based 3D printing

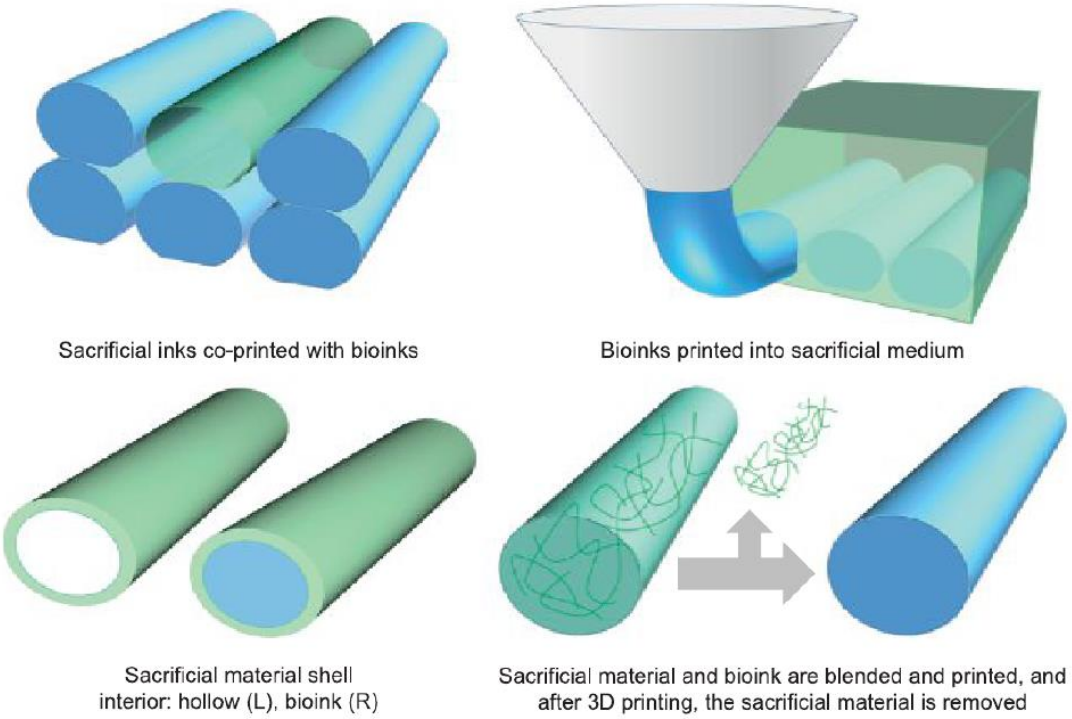




- Liquid bioinks are printed on a stage
- Gelation induced by UV/ thermal changes / baths with crosslinkers
- Need fast gelation after printing
- Cell sedimentation causes inhomogene structures

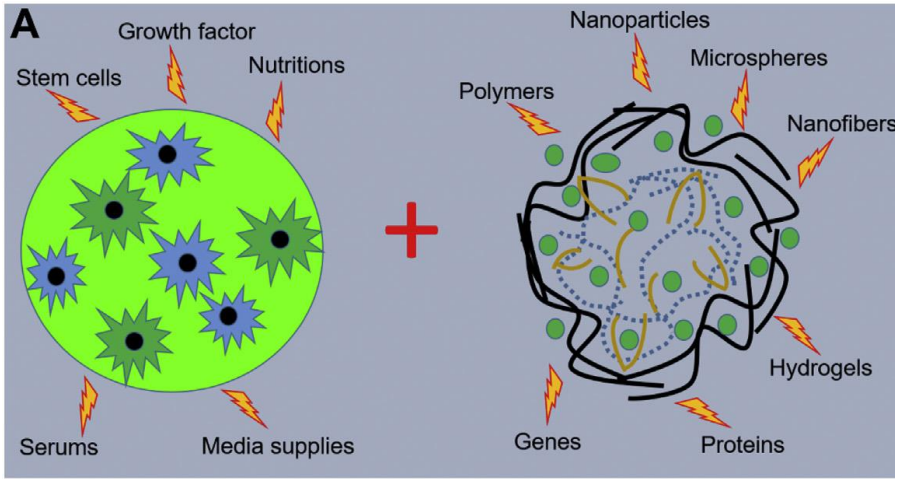
- viscous bioinks have enhanced structure fidelity
- Tunable viscosity with
  - hyaluronic acid
  - polymer fraction
  - polymer properties
  - degree of crosslinking

Sacrificial materials for structure support



- Co-printing bioink with sacrificial ink
- Sacrificial material builds a shell
- Mixture of bioink with sacrificial material to improve printing

Bioinks for extrusion based 3D printing



- Bioink consists of various biologics (cells, media, serum, genes, proteins etc.)

- natural polymers
  - alginate
  - hyalurnoic acids
  - silk fibroin
  - collagen gelatin

- synthetic polymers
  - polylactide-co-glycolide
  - polyethylene glycol
  - Poly-L-lactic acid
  - polycaprolactone

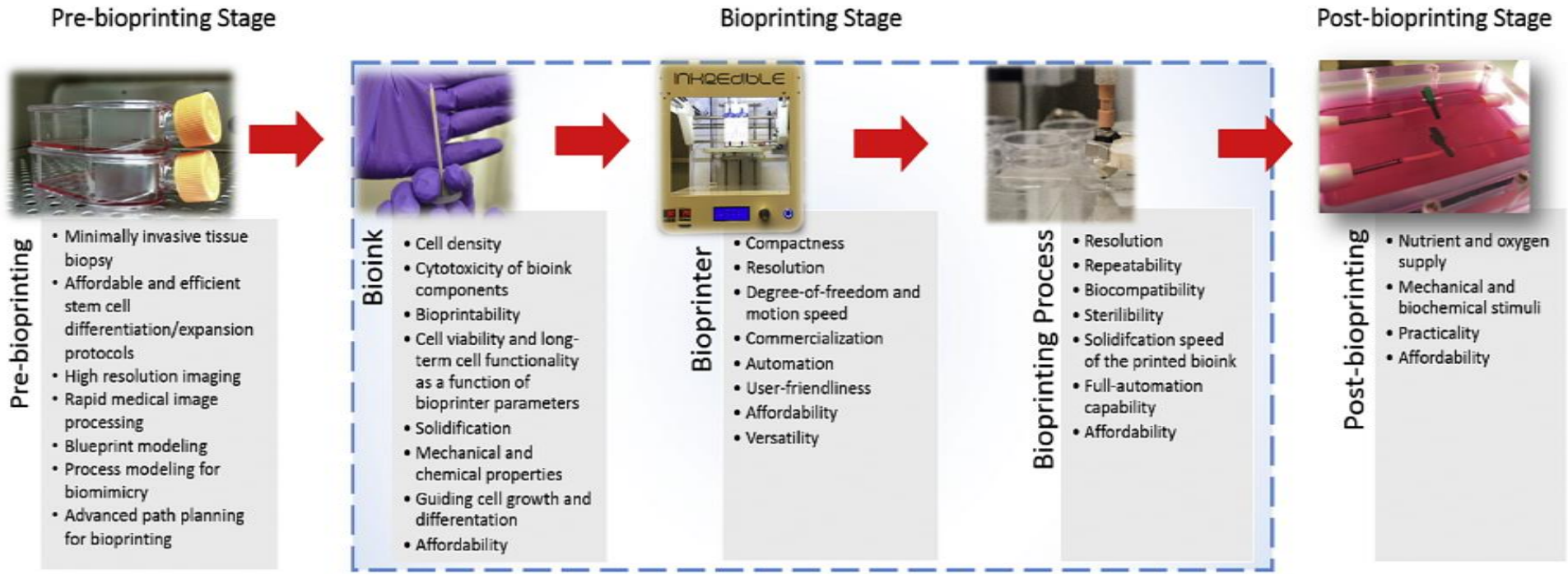
Table I. Relationships between bioink material properties and cell viability and behavior.

Bioink		Cells	
↑	Printing pressure	↓	Viability
↓	Nozzle diameter	↓	Viability
↑	Storage modulus	↓	Viability
↓	Degree of cross-linking	↑	Density in bioink
↓	Viscosity	↑	Density in bioink
↑	Degree of cross-linking	↓	Network formation
↑	Polymer fraction	↓	Network formation

- Bioink properties impact cells and cells can impact bioink properties

- cells impact bioink rheology and rheology impacts cell viablitily
  - ➢ characterize rheology in presence of cells

Challenges in bioprinting stages: pre-, post- and bioprinting



Cell viability is influenced by every parameter:

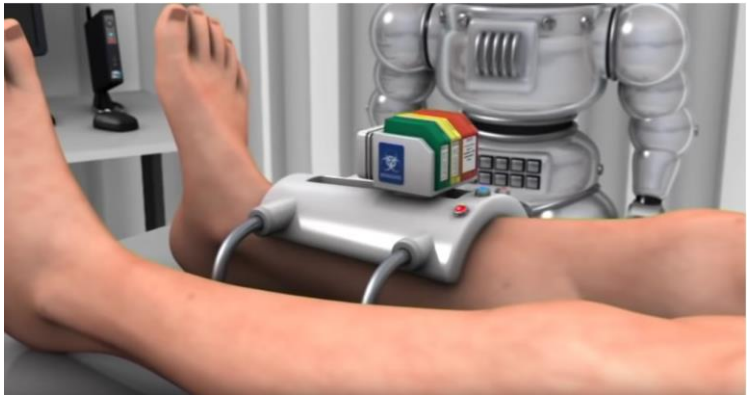
- **Bioink design** composition, rheological properties, cross-linking, degradation
- **printing parameters** pressure, duration, nozzle shape/diameter, cell type, concentration, sterility
- **post-printing** cross-linking, media supplements, culture conditions, removal of sacrificial material

➢ **influences function** proliferation, differentiation, cell alignment, tissue formation

Conclusion

- Important points in bioprinting
  - Cell encapsulation in the bioink
  - Appropriate gelation rate of ink
  - Suitable mechanical strength of ink
  - Elasticity which preserves cell viability and proliferation
  - Suitable bioprinting procedure

- Bioprinting is still limited and needs to be improved
  - Vessel formation
  - Gelation properties in physiological conditions
  - Well coordinated protocols
  - High quality and reproducibility products
  - Improved printers
  - Improved biomaterials
  - Lower prices
  - Ethics



https://www.azom.com/article.aspx?ArticleID=13810
https://www.youtube.com/watch?v=9D749wZSlb0

Article

Cell Reports

Modeling Tumor Phenotypes *In Vitro* with Three-Dimensional Bioprinting

COMMUNICATION

Cancer Modeling

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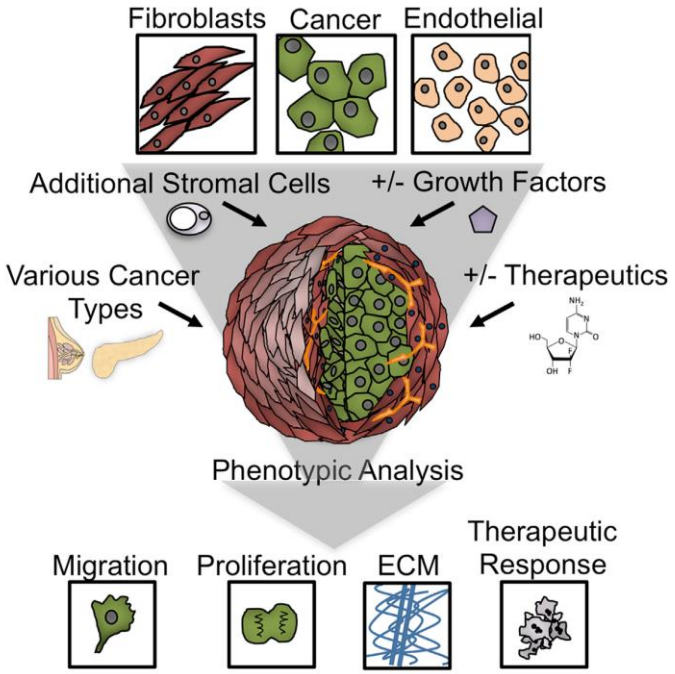
3D-Bioprinted Mini-Brain: A Glioblastoma Model to Study Cellular Interactions and Therapeutics

Marcel Alexander Heinrich, Ruchi Bansal, Twan Lammers, Yu Shrike Zhang, Raymond Michel Schiffelers, and Jai Prakash**

Modeling Tumor Phenotypes In Vitro with Three-Dimensional Bioprinting



Highlights

- Bioprinted tumor tissue is a scaffold-free model of tumor-stromal interactions
- Cells within bioprinted tissues mature, self-organize, and deposit matrix proteins
- Heterogeneity in therapeutic response, migration, and signaling can be assessed
- Primary patient tissue can be bioprinted into tissues for translational studies

Authors

Ellen M. Langer,
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Deborah G. Nguyen, Rosalie C. Sears

Method

Bioprint patient derived primary cells into scaffold-free in vitro tumor tissue to model patient specific tumors & microenvironment

Assessing cellular proliferation, ECM deposition, cellular migration, alterations in response to extrinsic signals or therapy etc.

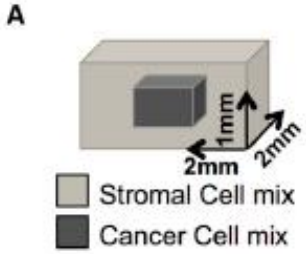
A cancer cell core surrounded by several stromal cell types to investigate ECM and self-organization

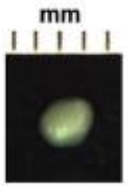
Printer: Organovo Novogen MMX Bioprinter

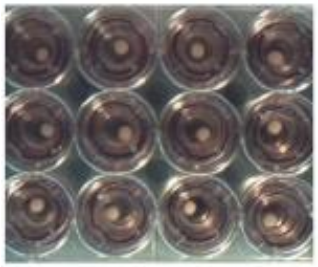
Bioink: thermally/chemically modified, tunable hydrogels to provide strength during printing and can be removed during culture to leave a purely cellular structure

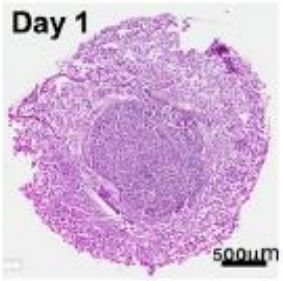
Cultured at standard culture conditions on transwell membranes

To model solid tumor architecture with tumor core and surrounding normal cells (tumor cells introduced 24h after bioprinting)





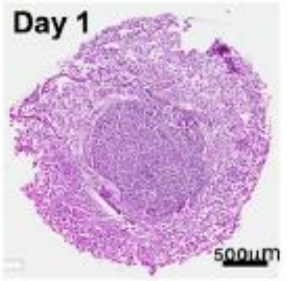




3D Bioprinting allows for generation of tumor models including multiple celltypes in a defined spatial Architecture

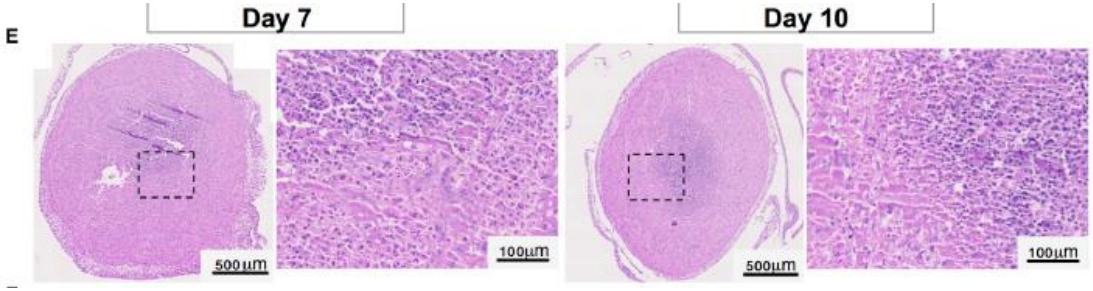
Modeling breast cancer: bioprinted tissue includes:

- estrogen receptor (ER)-postitive MCF-7 cells,
- primary human mammary fibroblasts (HMF)
- HUVECs

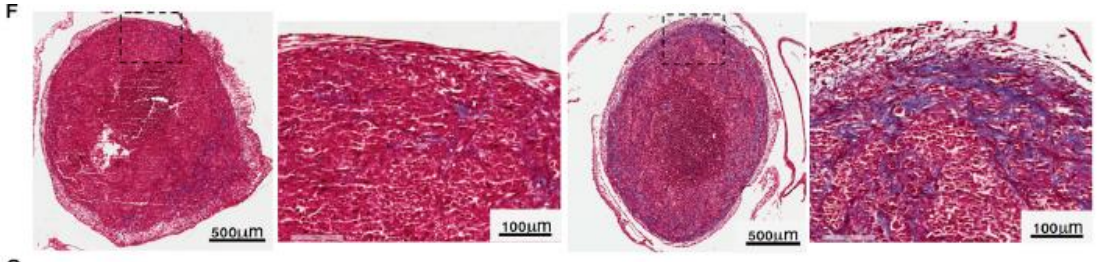


MCF7 cancer cells located in center surrounded by stroma

H&E shows tissue like cellular density and close interaction of epithelial and stromal cells



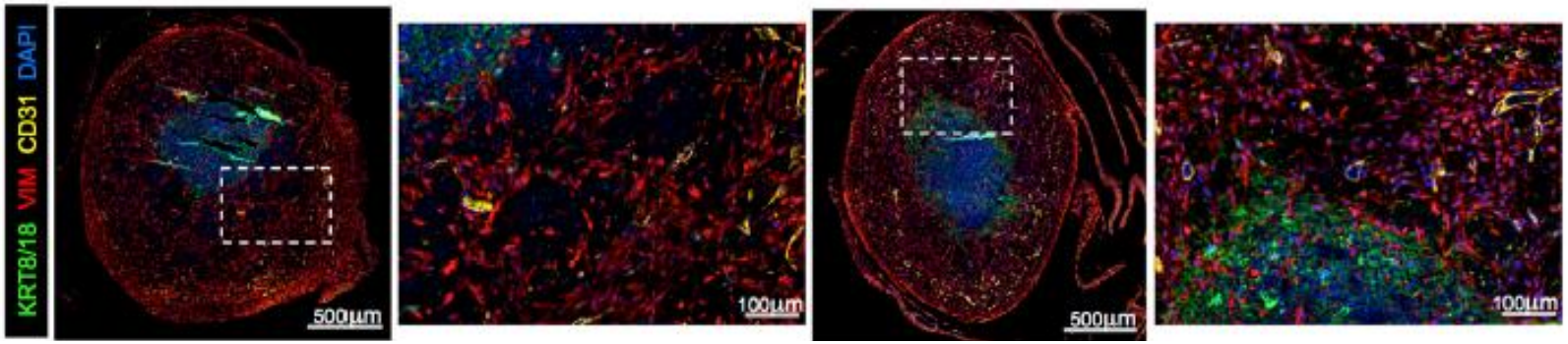
Masson`s trichrome staining shows collagen fibers in stromal compartment increasing over time → cells deposit ECM and mature



Interaction of cell types in bioprinted tumor model

Confirmation of close interaction by IHC

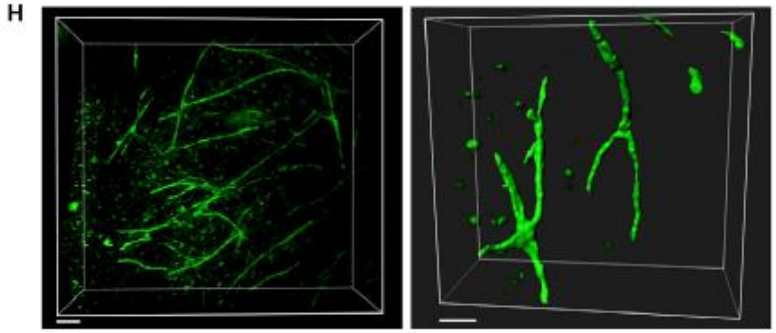
- Cancer cells KRT8/18
- Stromal fibroblasts VIM
- endothelial cells (PECAM1)



Endothelial cells self-organized into capillary like networks

Endothelial cells formed intact networks with multiple branch points

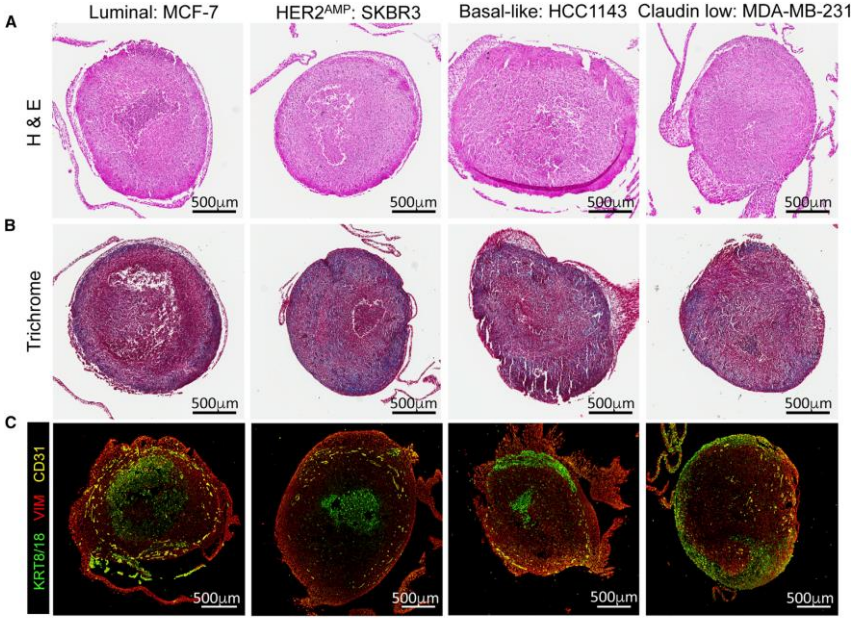
(CLARITY/ light-sheet microscopy, 3D reconstruction)

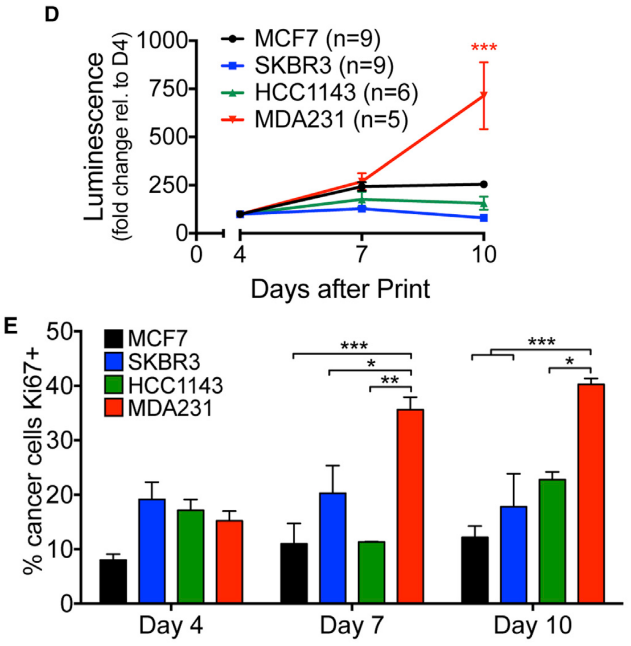


Bioprinted Tissues Can Model Distinct Tumor Cell Subtypes

Tumor heterogeniety can result from ex-or intrinsic signals and affect tumorigenic phenotypes – Is bioprinting a good model to study heterogeniety?

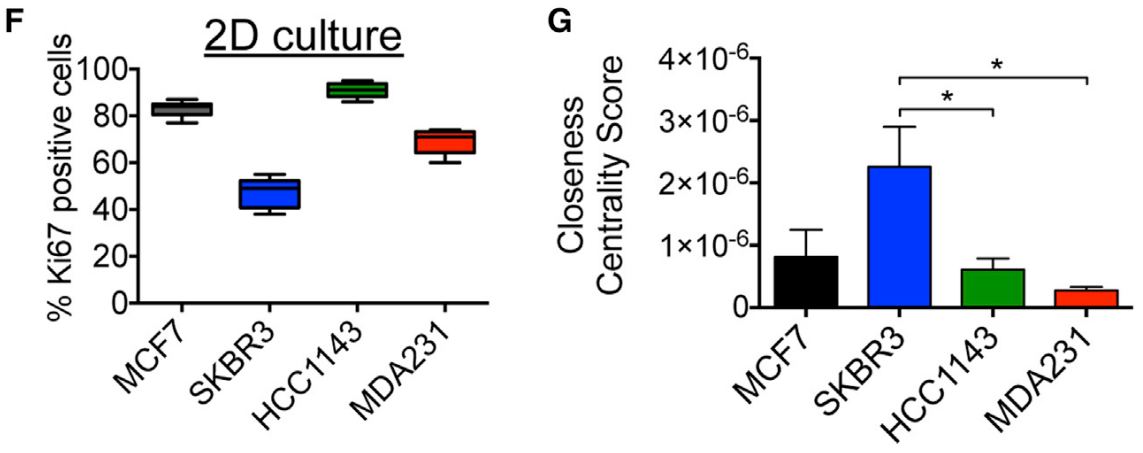
Bioprinting of breast cancer cell lines from distinct subtypes (luminal, HER2 amplified, basal-like and claudin low)





- To track growth:
  - Infection of cells with lentivirus encoding firefly luciferase
  - At days 4,7,10 add substrate containing D-luciferin
  - Claudin low cell line grew rapidly (increased luciferase readout and Ki67 positivity)

Bioprinted Tissues Can Model Distinct Tumor Cell Subtypes



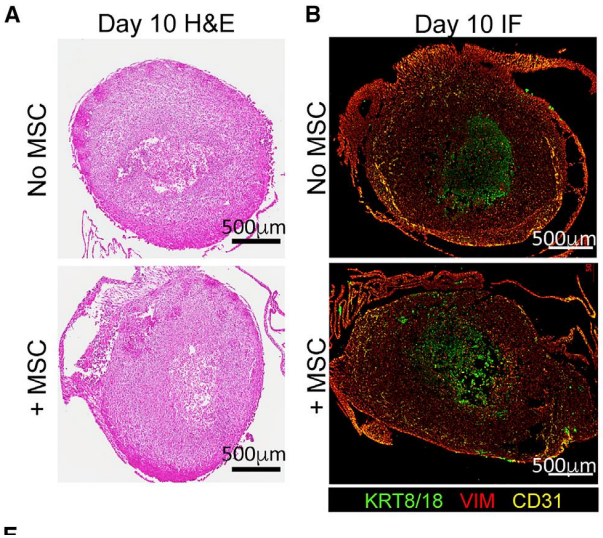
- Comparison of proliferative capacity in microenvironment and 2D culture
  - 2D culture showed much higher Ki67 positivity than 3D culture
- Closeness Centrality Score to show dispersal of tumor cells
  - High number = close proximity, low number = migration & dispersal
  - Claudin low subtype shows highest invasiveness

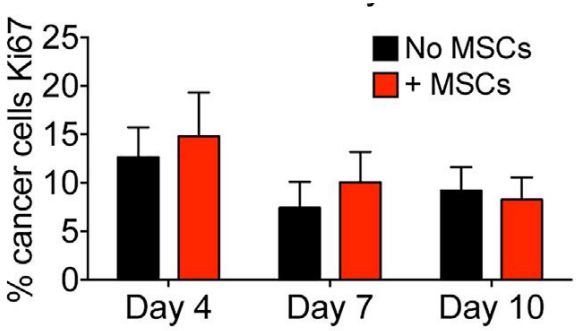
Bioprinted tissue can be used to analyze invasive phenotypes and model tumor subtypes

Distinct Microenvironments Can Be Modeled in Bioprinted Tissues

To model different microenvironments, additional tumor phenotype affecting cells were incorporated:

bone marrow derived MSCs – increase proliferation and migration via chemokines, cytokines, growth factors and ECM proteins

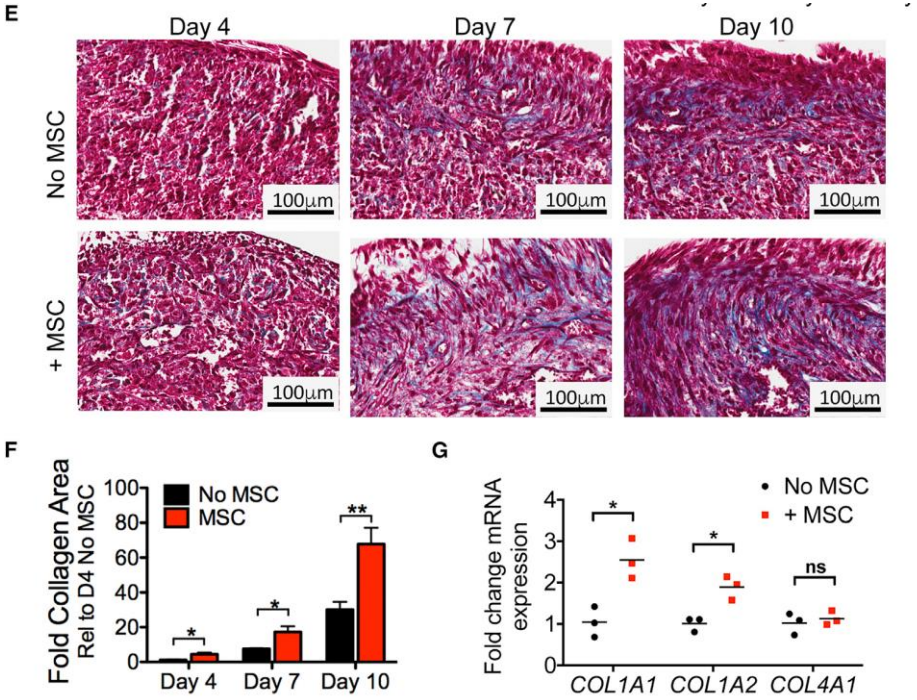




Similar histology to basic breast cancer prints → formation of cancer pocket

Quantification of Ki67 stainings do not show differences

Distinct Microenvironments Can Be Modeled in Bioprinted Tissues

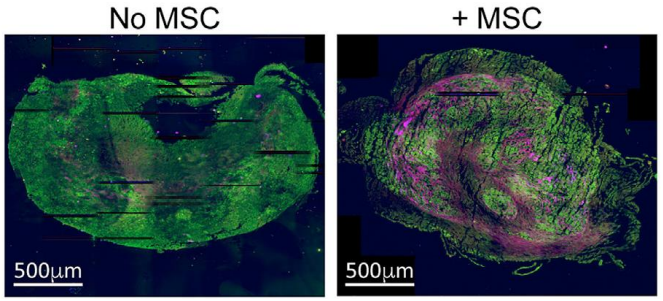


ECM deposition was increased in MSC containing 3D cultures at every timepoint

Increased collagen deposition indicates more reactive tumor microenvironment

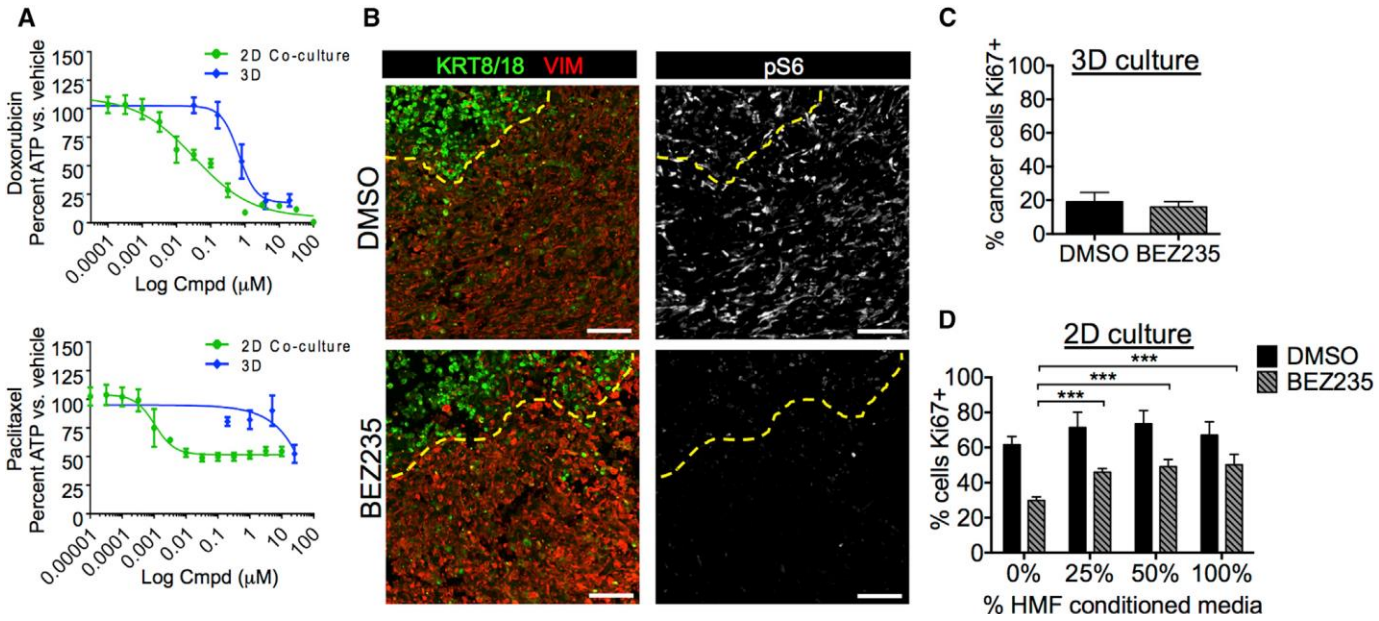
Increased expression of COL1A1, COL1A2 and COL1A4 mRNA in MSC containing bioprints

Increased load of mature collagen fibrils shown with second harmonic generation (SHG) imaging



MSCs contribute to reactive ECM rich tumor microenvironment which can be modeld by bioprinting heterotypic tumor tissue

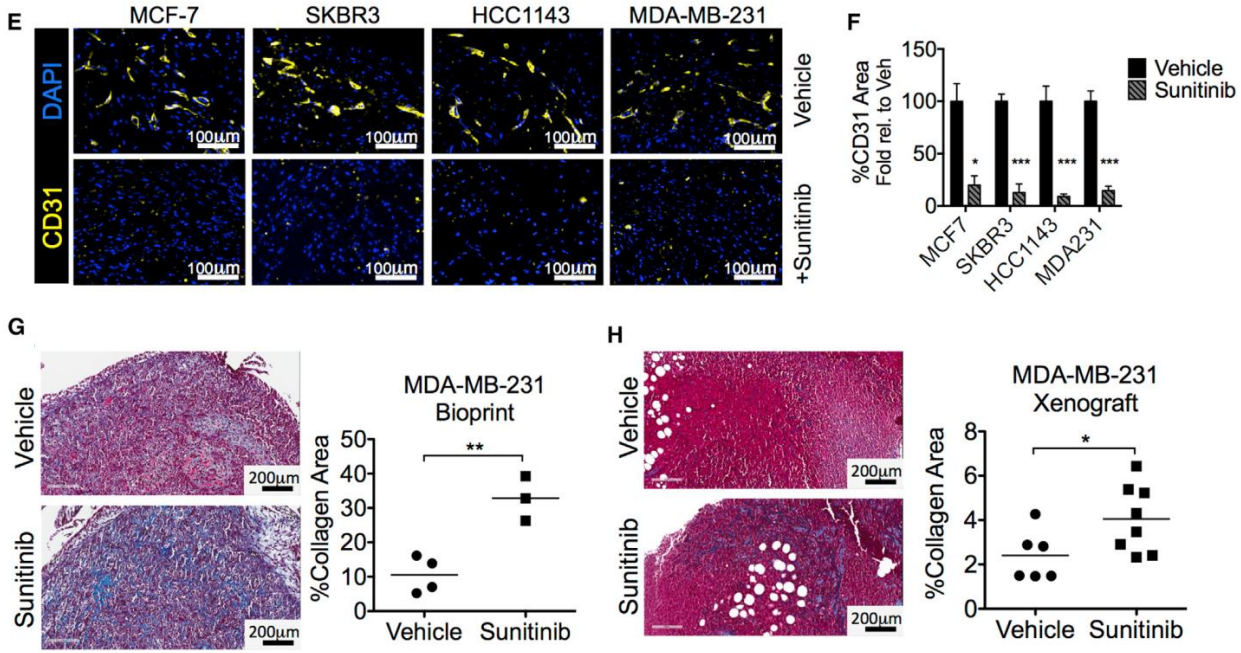
Bioprinted Tissues Can Be Utilized to Assess Therapeutic Efficacy



- 2D and 3D cultures with HMF, HUVEC, SPA, MCF-7 of same composition and density
  - 3 days daily treatment with chemotherapy (doxorubicin, paclitaxel)
  - Metabolic activity assessed with CellTiter Glo ATP utilization assay
  - Higher restistance to chemotherapy in 3D – ECM and cell organization may influence LD_{50}

- Bioprinting HCC1143 containing tissue treated with targeted therapy from day 4 (BEZ235, PI3K/mTOR inhibitor)
  - Reduction of phosphorylation of S6 ribosomal protein, but no reduction in $Ki67^+$ cancer cells
  - 2D culture with HMF conditioned medium before BEZ235 treatment shows decreased drug efficacy
  - paracrine factors from HMF contribute to therapeutic resistance in bioprinted tissue

Bioprinted Tissues Can Be Utilized to Assess Therapeutic Efficacy

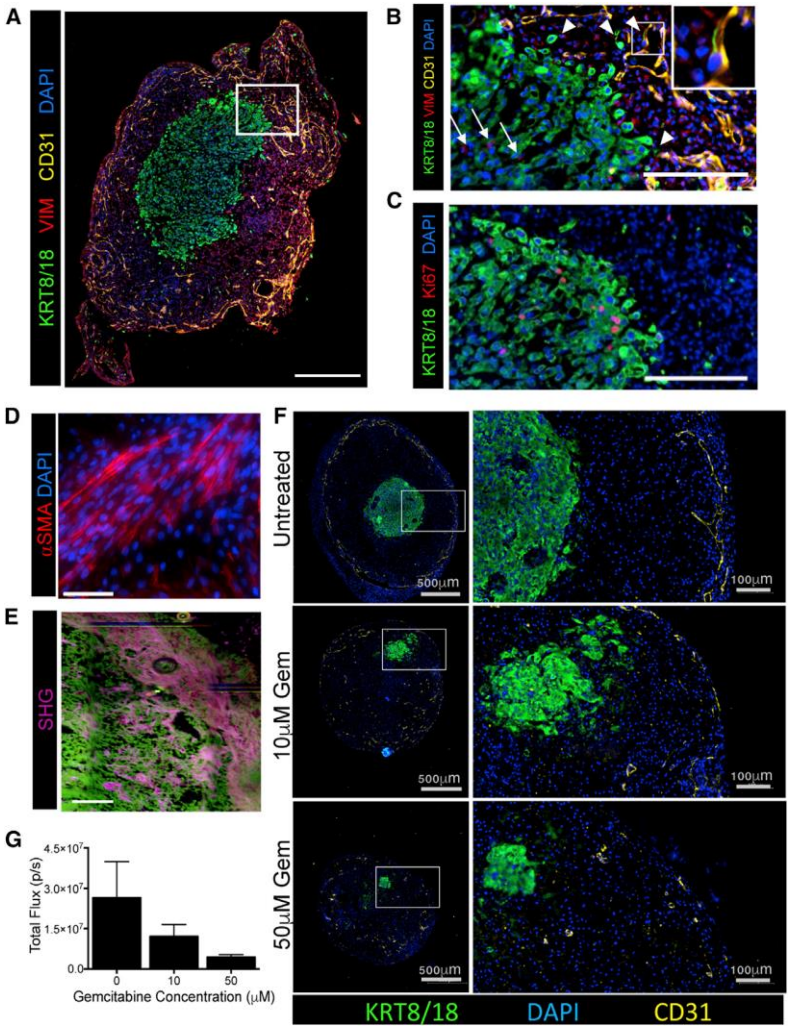


- Therapeutic targeting of stromal cells
  - 3D prints build vascular like networks in different breast cancer subtypes
  - 1uM Sutinib treatment on day 4 reduced endothelial networks
- anti-VEGF makes tumor more aggressive (via collagen deposition)
  - claudin low subtype treated with Sutinib shows increased collagen deposition – in vivio with orthotopic xenografts and in vitro in 3D cultures
- Bioprints recapitulate in vivio phenotypes

3D Bioprinting Can Model Additional Tumor Types (Pancreatic Cancer)

PDA shows large expansion of tumor microenvironment which can make up to 90% of tumor mass and contribute to progression and therapeutic resistance

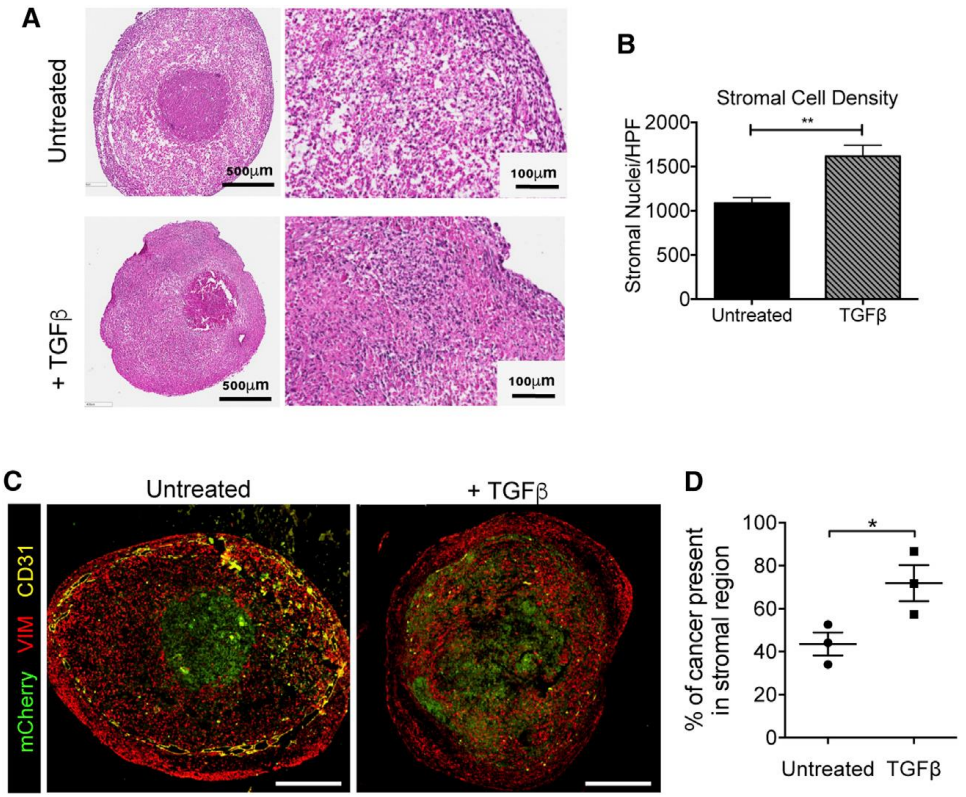
- Cancer cell line from patient-derived xenograft printed in stromal mixture of HUVEC and stellate cells
- At cancer stroma border, connection of cancer cells with endothelial cells seen
  - Cancer cells maintained proliferative capacity (Ki67)
- Bioprinted stellate cells express aSMA, as seen in desmoplastic tumors and contain mature collagen fibrils (SHG imaging)
  - dense activated stroma - similar to patients
- Treatment efficacy in PDA bioprints
  - Bioprinting of firefly luciferase expressing HPAFII cells with PSC and HUVEC treated with Gemcitabine on day 4
  - IF and luminescence readout at day 10 show dose dependent response to therapy



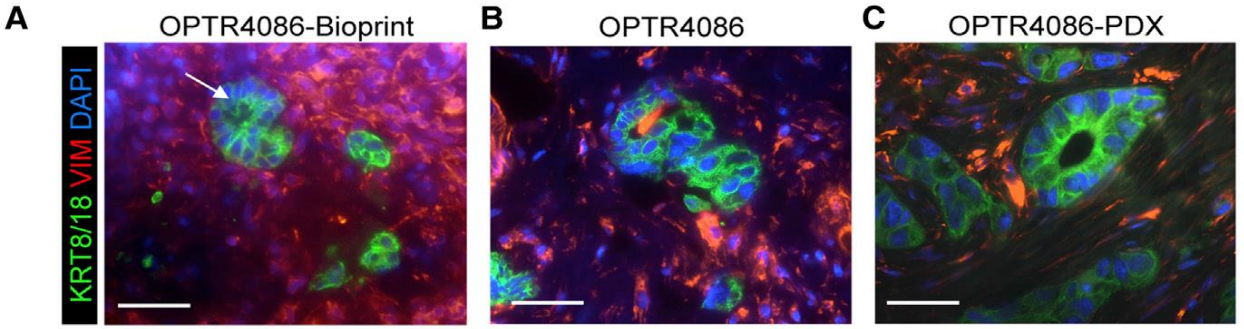
Cancer and Stromal Cells Respond to Microenvironmental Signals in Bioprinted Tumors

To examine cellular resonses within biprinted tissues to extrinsic signals known to alter tumor phenotypes in vivo

- Treatment of bioprinted pancreatic cancer tissue with TGFβ
  - TGFβ increased cellular densitiy of tissue
- TGFβ can affect tumor cell intrinsic migratory capacity
  - Untreated tumor cells remained in central region
  - Cytokine-treated tumor cells showed disrupted border and migration into surrounding stroma



Bioprinted Tissues Generated from Primary Patient Tumors Recapitulate In Vivo Morphology

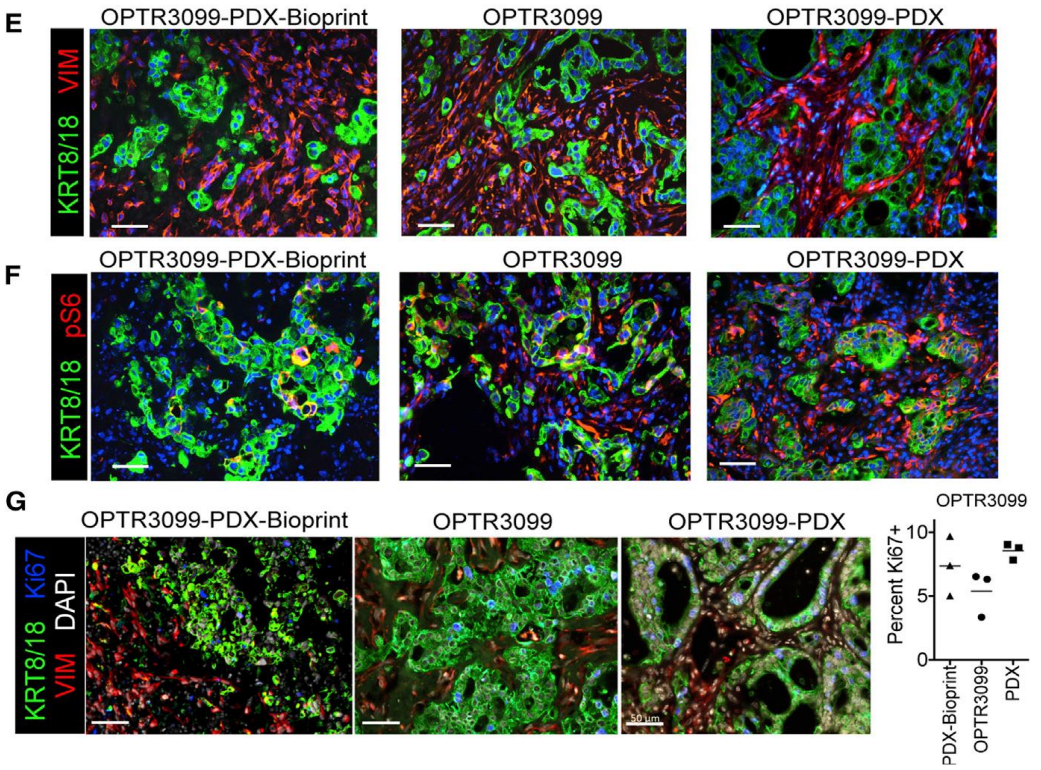


- Dissociated patient tumor tissue bioprinted with surrounding HUVEC and PSC, Cultured for 6-10 days
- Tumor sample of patient
- Patient derived xenograft (PDX)

- Bioprinted cancer cells show high order structure
- Some regions display a cuboidal organisation surrounding a lumen
  - similar to structures found in the primary patient and PDX tumor tissue

Bioprinted Tissues Generated from Primary Patient Tumors Recapitulate In Vivo Morphology

- Bioprinted, patient tumor and PDX tissue show similar morphology
- Spacial organization similar to in vivo tissue
- Bioprinted tissue recapitulates signaling heterogeniety
  - Similar pS6 and mTOR signaling readout to PDX and patient tumor
  - Bioprinted tissue has low pS6 staining near edges
- Levels of proliferation in bioprinted tissue matching in vivo conditions



Conclusions

- Accurate model of human tumors
- allowance of integration of addintional cell types into 3D culture
- Signifincantly altered gene expression and tumorigenic phenotypes compared to 2D cultures
- Spatially defined architecture
- Purely cellular model with tissue-like organization
- Models therapeutic response in vivo

- Prognositc use of bioprinted tumors
- Inclusion of patient matched stromal cells

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3D-Bioprinted Mini-Brain: A Glioblastoma Model to Study Cellular Interactions and Therapeutics

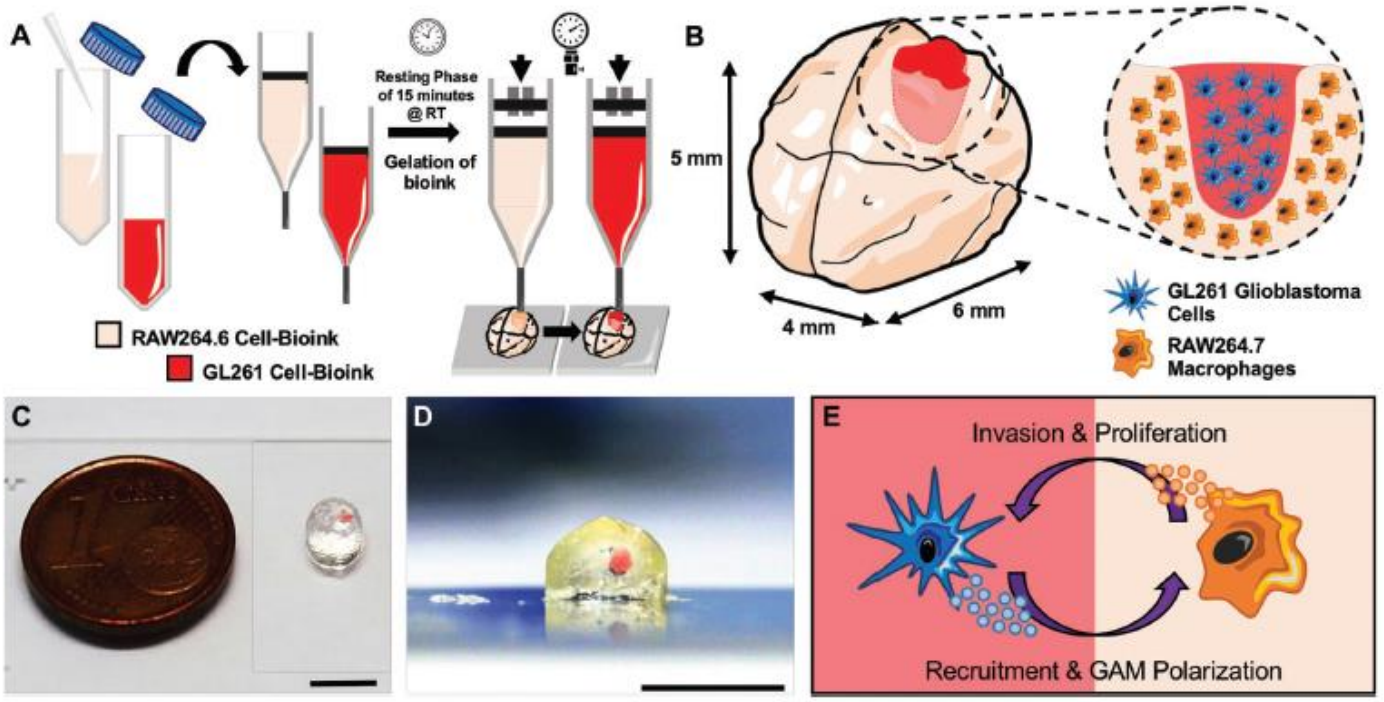
Marcel Alexander Heinrich, Ruchi Bansal, Twan Lammers, Yu Shrike Zhang, Raymond Michel Schiffelers, and Jai Prakash**

- Creation of a 3D bioprinted «mini-brain»
- Mimic the interaction of GBM associated macrophages and GBM cells, cultured under conventional cell culture conditions
- Enables cells to reorganize and interact
  - For phenotypic alteration studies
  - For use in therapeutic efficiacy studies
- Not stem-cell originated real tissue model

Method

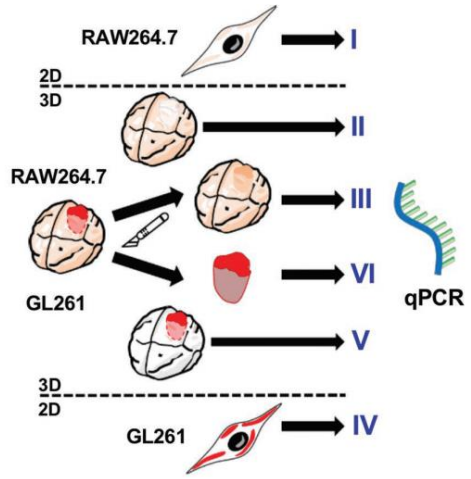
- Bio-Ink: gelatin methacryloyl (GelMA) for biocompatibility and shear-thinning with gelatin for stability
  - Pore size of crosslinked gel allows free cell migration and movement

- 2-step bioprinting process for well defined tumor location
  1. Larger brain model with mouse macrophages and empty cavity
  2. Filling of cavity with mouse GBM cells
  3. Photo-crossliniking of the construct



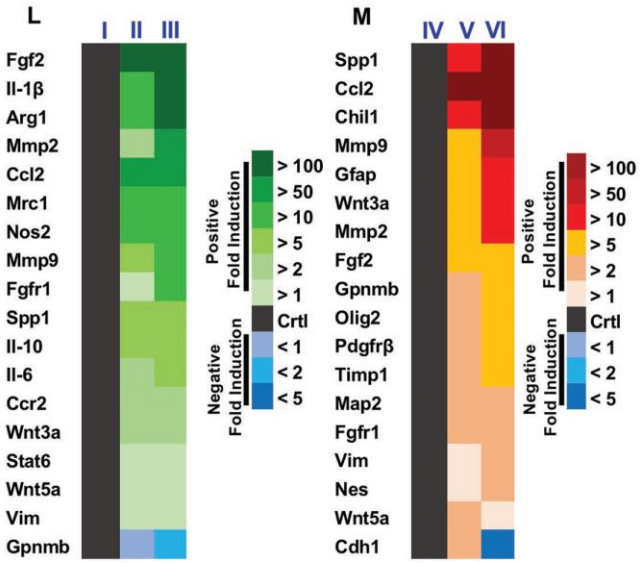
Effects of direct crosstalk between cells in the mini-brain on transcriptomic level

Bioprinting of mini-brains with macrophages and a cavity filled with GBM cells – mimicking realistic microenvironment. GBM resection and analysis after 4 days of culturing.



Schematic of the experimental setup for bioprinted RAW/GL co-culture model

Heat map of expressed genes in macrophages and GBM cells



RAW264.7:
I 2D monolayer
II 3D single bioprinted
III 3D co-cultured with GL261

GL261:
IV 2D monolayer
V 3D single bioprinted
VI 3D co-cultured with RAW264.7

Macrophages show overexpression of GAM specific markers (Fgf2, Il-1b, Arg1, Nos2, Fgfr1, Il-10, Il-6, Mmp2,Mmp9) in co-culture

GBM cells show overexpression of GBM markers (Gfap, Chil1, Olig2, Pdgfrb, Timp1, Spp1) in co-culture

GBM cells show characteristics of EMT e.g. increase in Vim & Nes, decrease of Cdh1 → **GBM cells show migratory characteristics**

This in vitro model resembles the characteristics found in GBM in vivo to a great extend.

Conclusions

- These results suggest:
  - corsstalk between GAM and GBM cells has effect on tumor growth and inhibition of GAM can result in reduction of tumor growth

- Mini-brains can:
  - replicate the clinical situation
  - be used to reduce and refine animal experiments
  - Be used to mimick TMEs of various tumors
  - Be used to investigate therapeutic approaches (rapid drug screening)

- In future, complexity of this model can be increased by adding other TME components e.g. astrocytes

Thank you for your interest!

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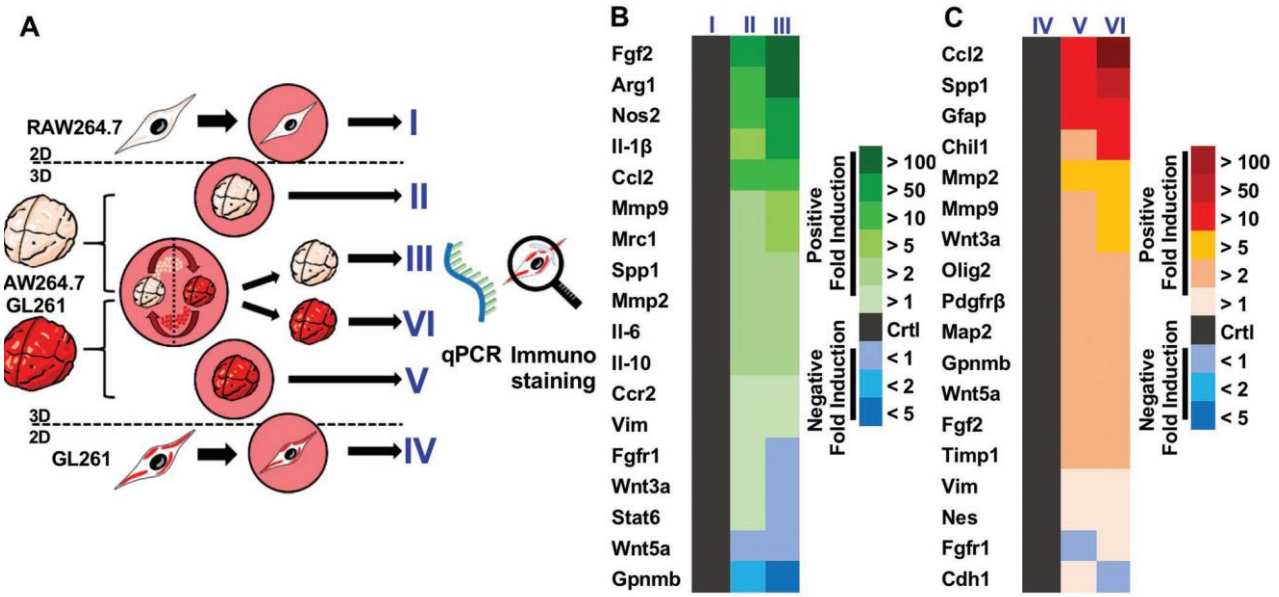
Langer et al. Modeling Tumor Phenotypes In Vitro with Tree-Dimensional Bioprinting. Cell Reports (2019)

Investigation of paracrine signaling between separately bioprinted RAW264.7 and GL261 cell-laden mini-brains in co-culture

Both cell lines survived in the mini-brain with high metabolic activity for at least 10 days



Gene expression in RAW264.7:
I 2D monolayer
II 3D single bioprinted
III 3D co-cultured with GL261

Gene expression in GL261:
IV 2D monolayer
V 3D single bioprinted
VI 3D co-cultured with RAW264.7

Mini-brains only loaded with one cell type cultured in same well to investigate interactions via growth factors and secreted cytokines only

Compared to 2D cultures: ECM remodeling enzymes (Mmp2, Mmp9)
GAM phenotypic markers (Fgf2,Il-1b, Arg1)
GBM specific markers (GFAP, Chil1)

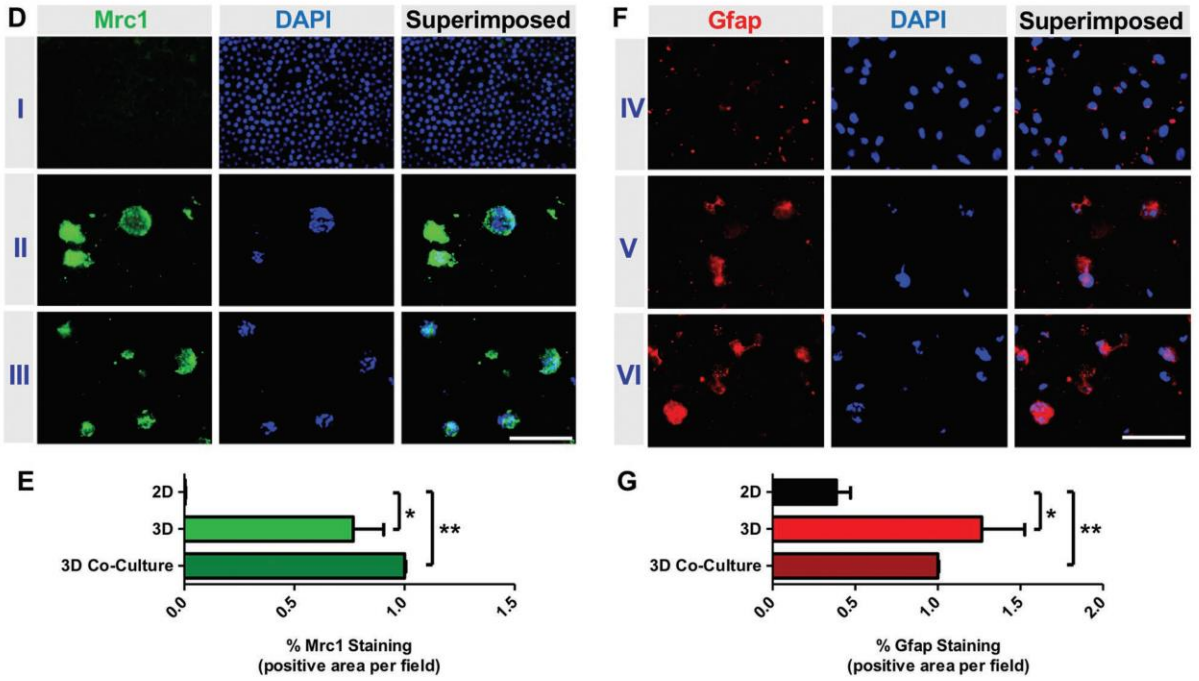
Co-culture vs. Single RAW: GAM markers (Fgf2,Il-1b, Arg1, GFAP, Chil1)
markers involved in recruitment and polarization (Ccl2, Spp1)

3D mini-brains could recapitulate phenotypic characteristics of cells found in GBM in vivo (and are superior to 2D culture)

ICH: Mrc1 and Gfap (most common markers of GAMs and GBM cells in vivo)

4 days post bioprinting: 3D culure compared to 2D culture
3D single bioprinted vs. 3D co-cultured

RAW264.7:
I 2D monolayer
II 3D single bioprinted
III 3D co-cultured with GL261



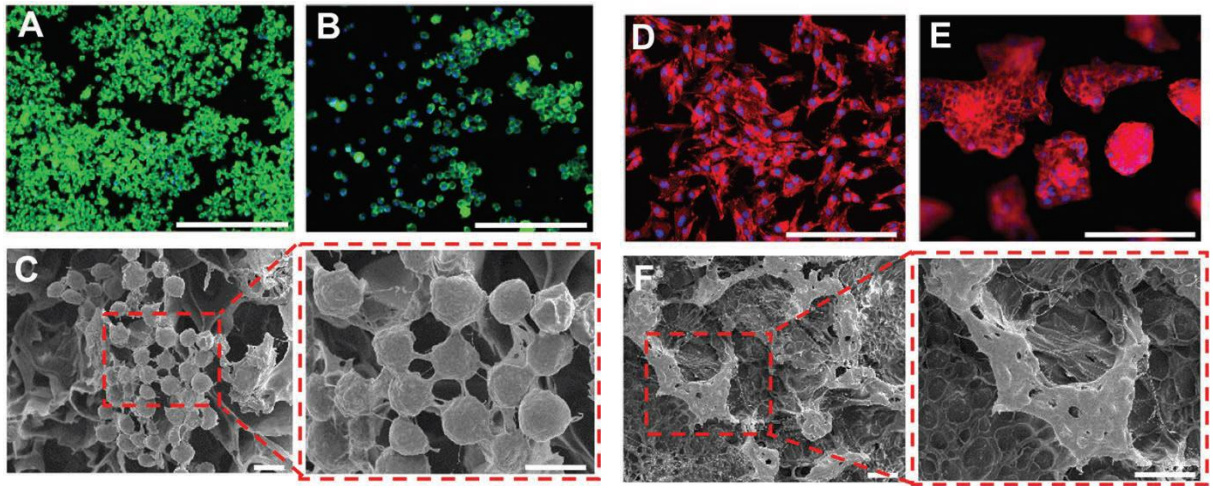
GL261:
IV 2D monolayer
V 3D single bioprinted
VI 3D co-cultured with RAW264.7

scale bar = 100 µm; mean + SEM; two-tailed, unpaired t-test

Migration and juxtacrine signaling between macrophages (RAW264.7) and GBM (GL261) cell-laden mini-brains in direct cell-to-cell contact

Cell migration relies on ability of cells to attach to surface:

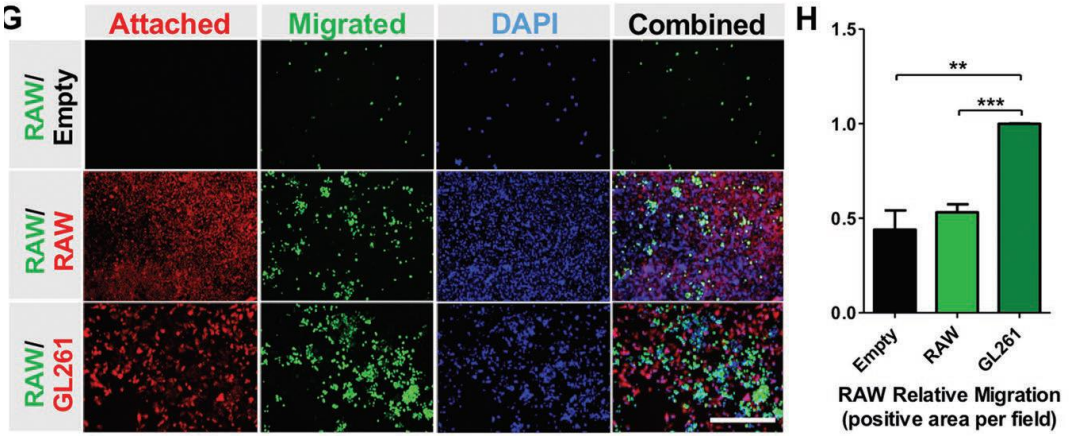
comparison of cell attachment onto cell culture plate vs. bioink matrix



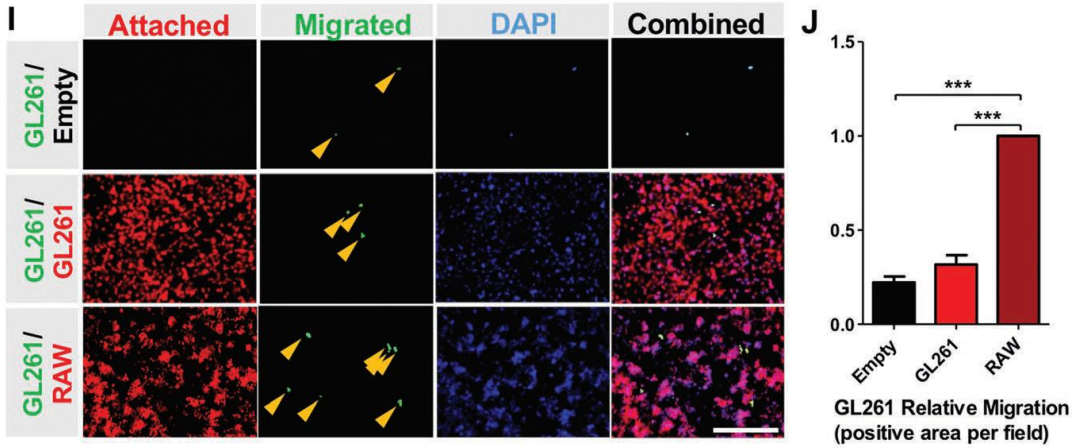
F-actin/DAPI stainings for macrophages (left) and GBM cells (right) show attachment to bioink matrix

Migration and juxtacrine signaling between macrophages (RAW264.7) and GBM (GL261) cell-laden mini-brains in direct cell-to-cell contact

Migration assay: placing bioprinted cell-laden mini-brains on top of a monolayer of opposite cells. CMFDA labelled cells towards empty, same cells, opposite cells



Macrophage migration assay
4 days of culture, 24h contact:
More macrophages migrated toward GBM cells compared to empty wells or themselves. → GBM cells actively recruited macrophages



GBM cells migration assay
10 days of culture, 24 h contact:
GBM cells were less migratory but still higher migration towards macropghages then empty wells or themselves.

scale bar = 100 µm; mean + SEM; two-tailed, unpaired t-test

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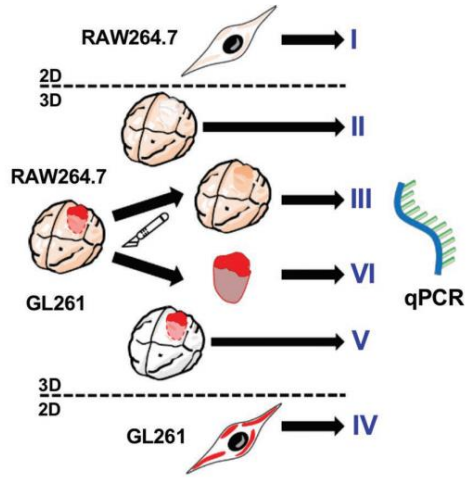
Bioprinting of mini-brains with macrophages and a cavity filled with GBM cells – mimicking realistic microenvironment. GBM resection and analysis after 4 days of culturing.



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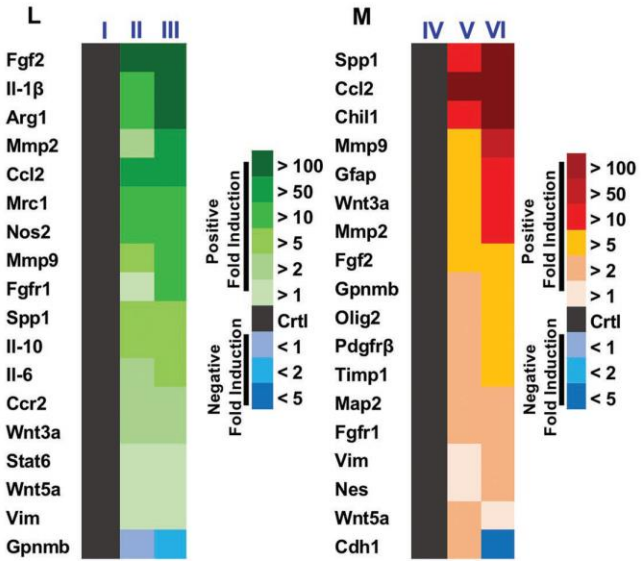
Heat map of expressed genes in macrophages and GBM cells



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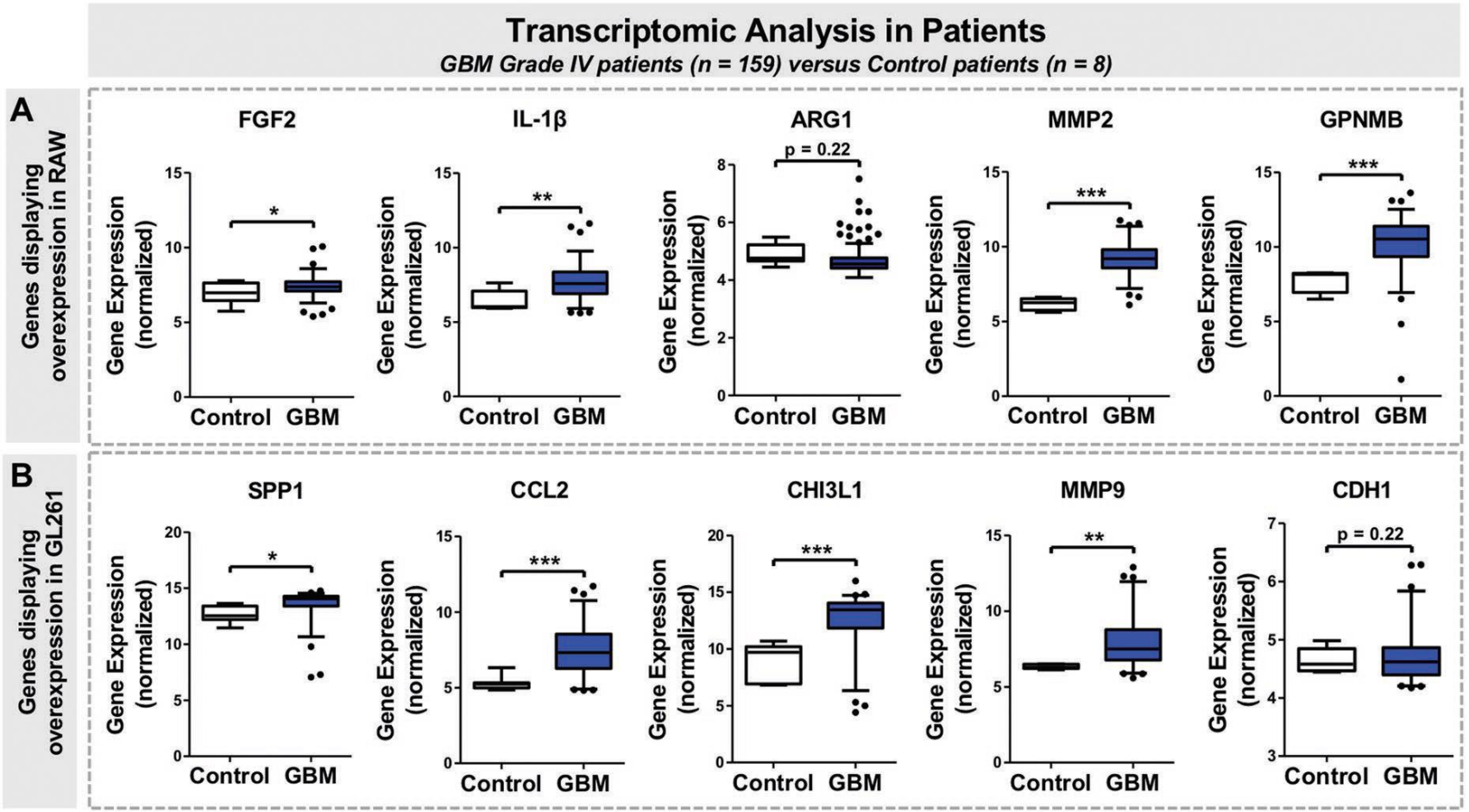
GBM cells show characteristics of EMT e.g. increase in Vim & Nes, decrease of Cdh1 → **GBM cells show migratory characteristics**

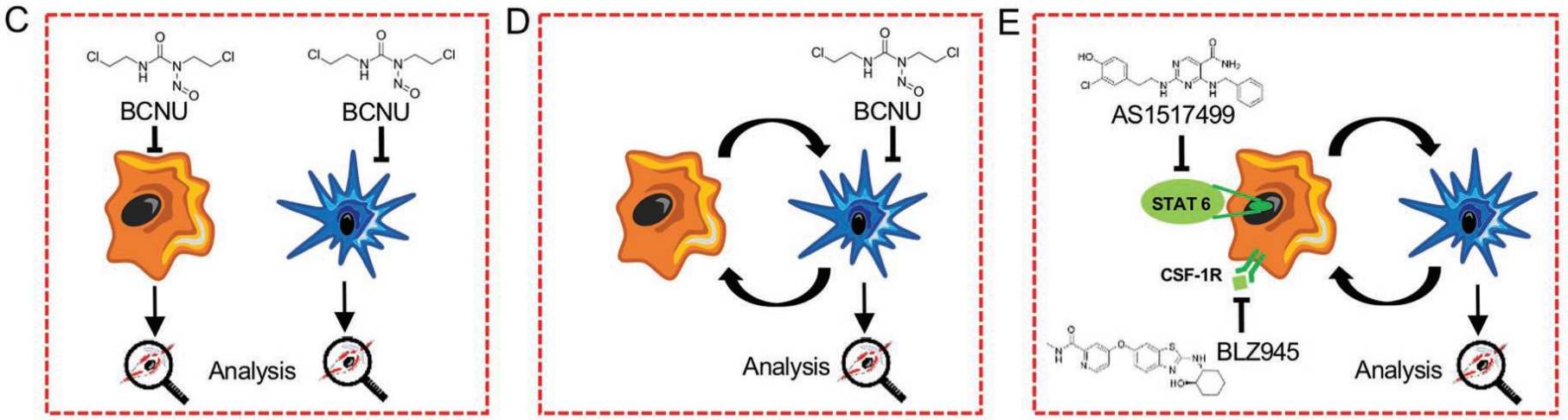
This in vitro model resembles the characteristics found in GBM in vivo to a great extend.

Confirmation of clinical relevance of upregulated genes in the mini-brains



Transcriptomic analysis of publicly available data from 159 GBMs versus 8 controls.
Several of the upregulated markers in mini-brains are upregulated in GBM patients (except Arg1).
GPNMB and CDH1 are downregulated in the mini-brain.

Analysis of immuno- and chemotherapeutic drug compounds in bioprinted mini-brains

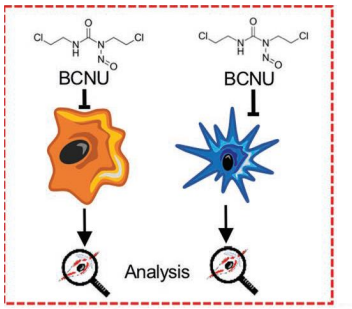


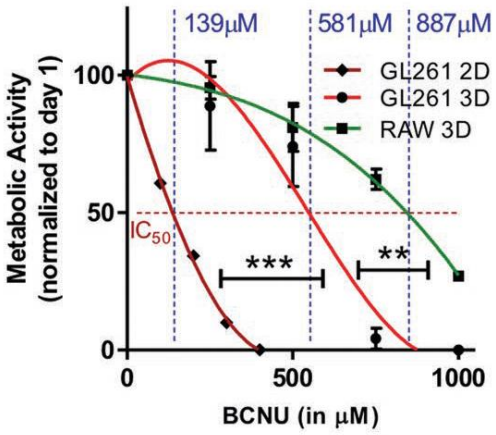
Chemotherapeutic BCNU (carmustine) on monoculture mini-brains of macrophages or GBM cells.
Treatment from d4-d6, followed by metabolic activity assay (Alamar blue assay).

BCNU treatment (d4-d6) on GBM pieces out of co-culture mini-brains. Followed by metabolic activity assay.

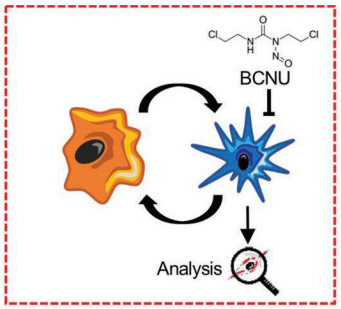
Immunomodulatory agents BLZ945 (Csf-1 inhibitor) for GBM and Stat6 inhibitor (AS1517499) for macrophages on co-culture mini-brains. Treatment on d1 & d3, followed by culturing of isolated tumor pieces (to d6) and metabolic activity assay.

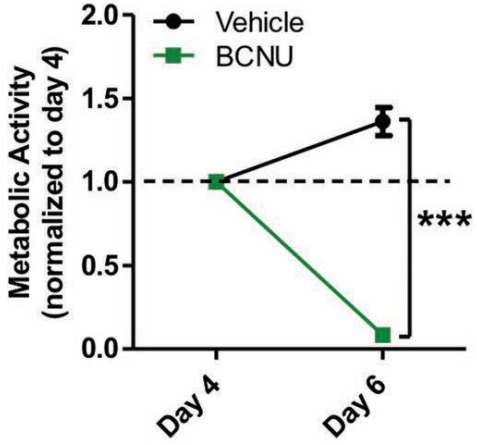
Analysis of immuno- and chemotherapeutic drug compounds in bioprinted mini-brains: Metabolic activity assays



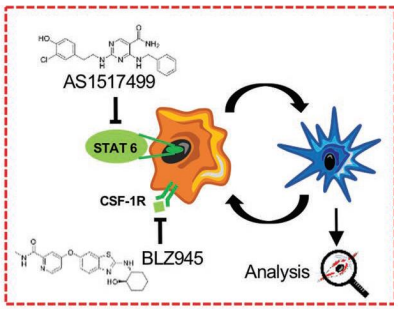


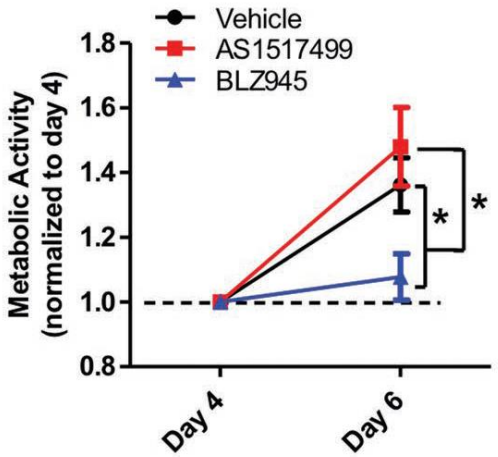
IC_{50} of 3D monocultured GBM cells is higher than 2D cultured GBM cells.
IC_{50} of macrophage monoculture indicates high resistance to BCNU treatment.





Tumors isolated from co-culture show higher growth rate, BCNU strongly inhibited growth compared to monocultured mini-brains.

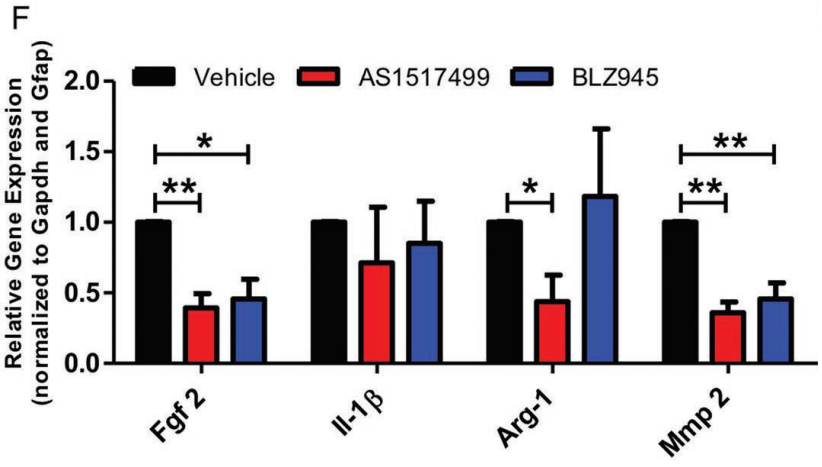




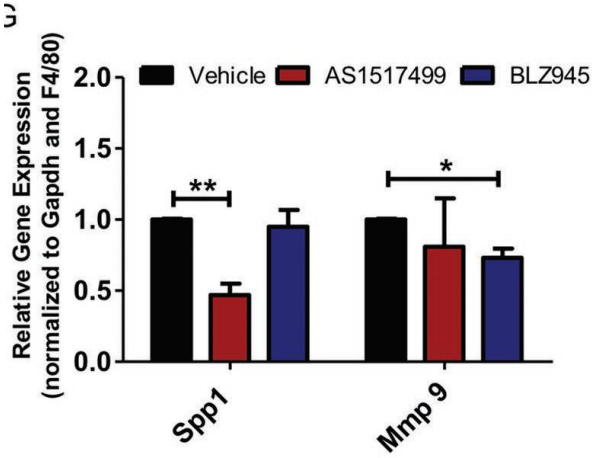
Tumors isolated from co-culture, treated with BLZ945 showed slower growth compared to vehicle treated tumors.

Gene expression of GBM markers for macrophages and tumor cells

Gene expression for selected genes in **macrophages** after 4 days of culture. Treatment on day 1 and day 3 post-bioprinting.



Gene expression for selected genes in **GBM cells** after 4 days of culture in mini-brains. Treatment on day 1 and day 3 post-bioprinting.



AS1517499 downregulated Fgf2,Arg1 and MMp2 in macrophages.

Spp1 and Mmp9 expression is downregulated in GBM cells after treatment.

Conclusions

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