Cartilage TE: *in vitro* and *in vivo* models and assays

Module 3, Lecture 5

20.109 Spring 2008

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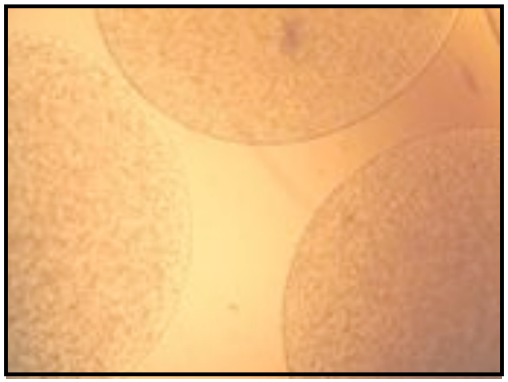
Topics for Lecture 5

- Module 3 overview: week 3
- Gene and protein expression assays
- Models and assays *in vivo*
- Clinical relevance (if time today)

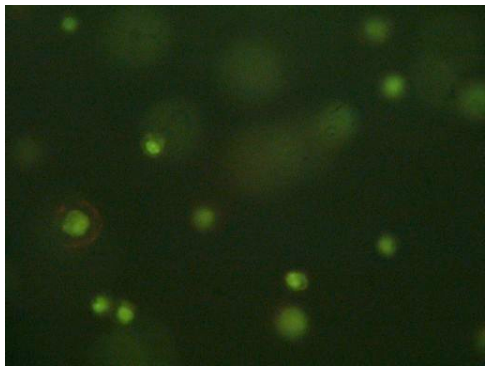
Module overview: lab

Day 1: design

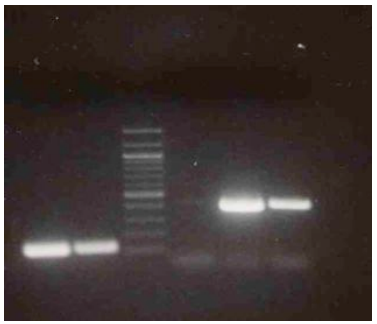
Day 2: seed cultures



Day 3: viability assay



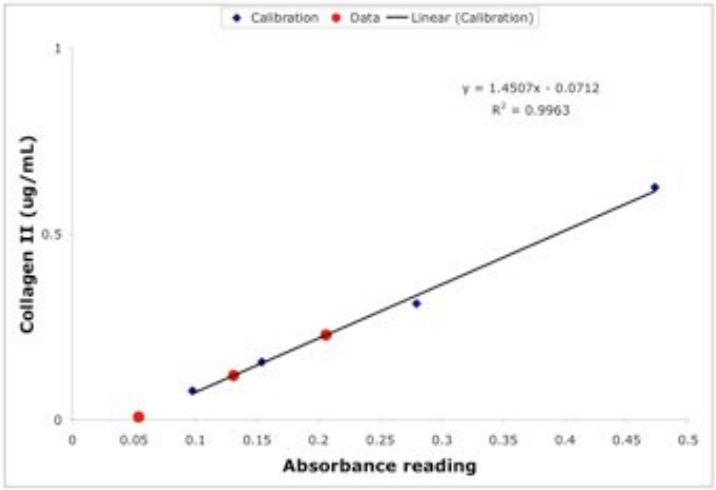
Day 4: prep RNA+cDNA



Day 5: transcript assay

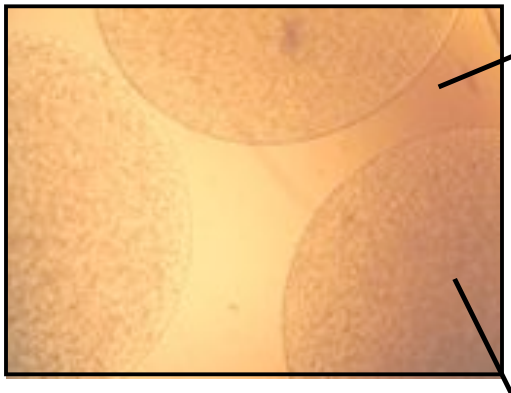
Day 6: protein assay

Day 7: remaining analysis



Day 8: your research ideas!

Module overview: week 3



1. Collect supernatant

Test for collagen proteins (by ELISA)

2. Collect and lyse cells

Purify mRNA from cells → Amplify collagen cDNAs →

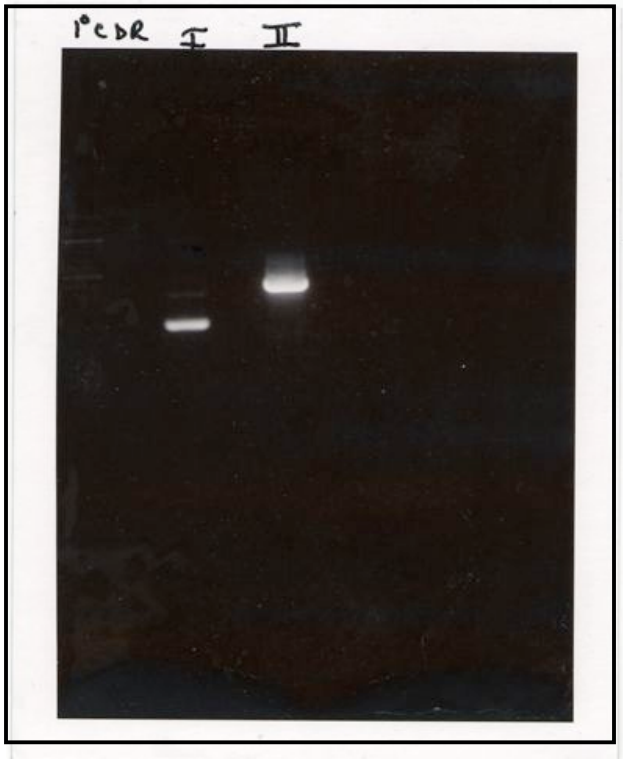
Compare collagen I and II transcript levels

Day 5: transcript analysis

Last time: amplified COL1A1 and COL2A1 cDNAs from cellular RNA isolates

Today: run cDNA on gel, compare intensities

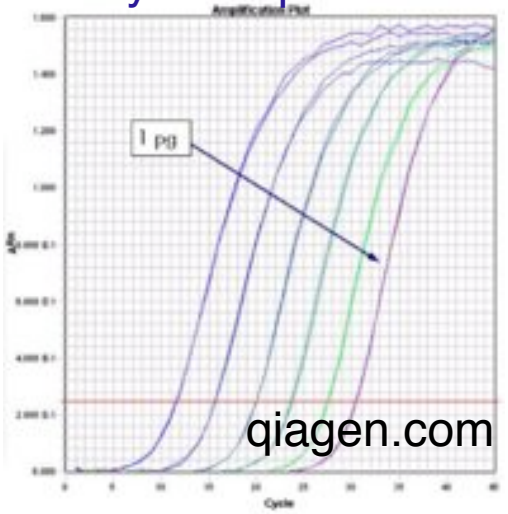
- low dynamic range for DNA levels on gel
- potential loading variability
- what controls, changes would improve our assay?

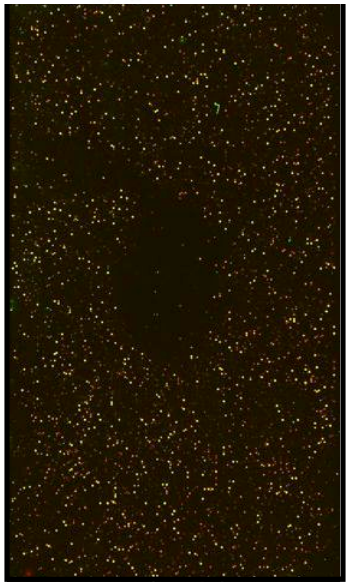


Transcript-level assays

- RT-PCR (end-point)
  - advantages: simplicity, can be semi-quantitative
  - to quantify: co-amplify housekeeping gene
- q-PCR (real-time)
  - uses fluorescent DNA probes
  - advantages: quantitative, potential for multiplexing
  - quantitation may be done several ways
    - standard curve, with housekeeping normalization
    - efficiency-correction, allows comparison between genes
    - absolute quantification possible with radiolabeling
- Microarrays
  - advantages: high throughput (potential for genome-wide)
  - compare two experimental conditions using different fluorophores to tag the mRNAs
  - requires specialty equipment, more expensive
  - more complicated analysis (hence use of standards)

Why PCR plateaus?

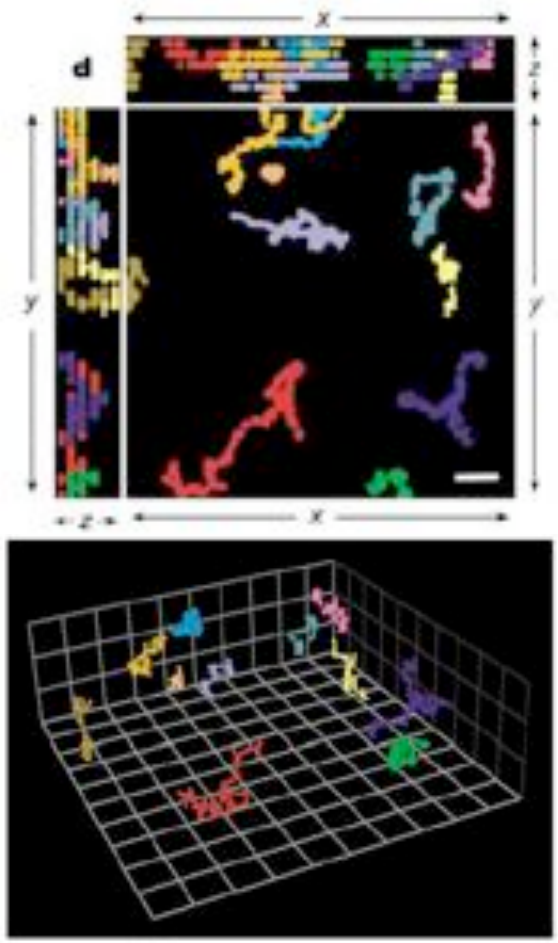




Current Protocols in Cell Biology, Molecular Biology

Day 5: image analysis

- Imaging data is often high throughput
  - potentially 4D: time-lapse, *xyz*
  - computation is required to extract meaningful results
  - human design and interpretation of analysis is also necessary
- Many commercially available analysis packages
  - specialty packages may run $20-30K
  - NIH ImageJ freely available
- Your analyses (Day 5+6)
  - relative intensity and/or size of cDNA bands
  - automated counting of live cell populations
  - optionally, explore other features



Images from: T.R. Mempel, et al. *Nature* **427**:154 (2004)

What kinds of information can imaging provide?

- Static fluorescence intensities for cells
  - viability, apoptosis, or other cell state
  - staining of cytoskeleton or organelles
  - labeled antibodies can indicate presence of a certain receptor on cells, phosphorylated molecules, etc.
- Dynamic fluorescence intensities for cells
  - calcium (or other) fluxes using indicator dyes
  - tracking cell motility in different matrices
- Different images modalities provide different information
  - fluorescence vs. MRI
  - resolution, depth, coverage, signal:noise, etc.
- What do we learn from single-cell vs. population assays? What are the drawbacks of each?

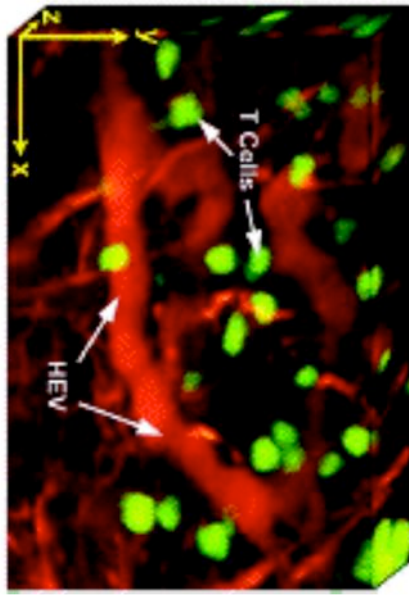
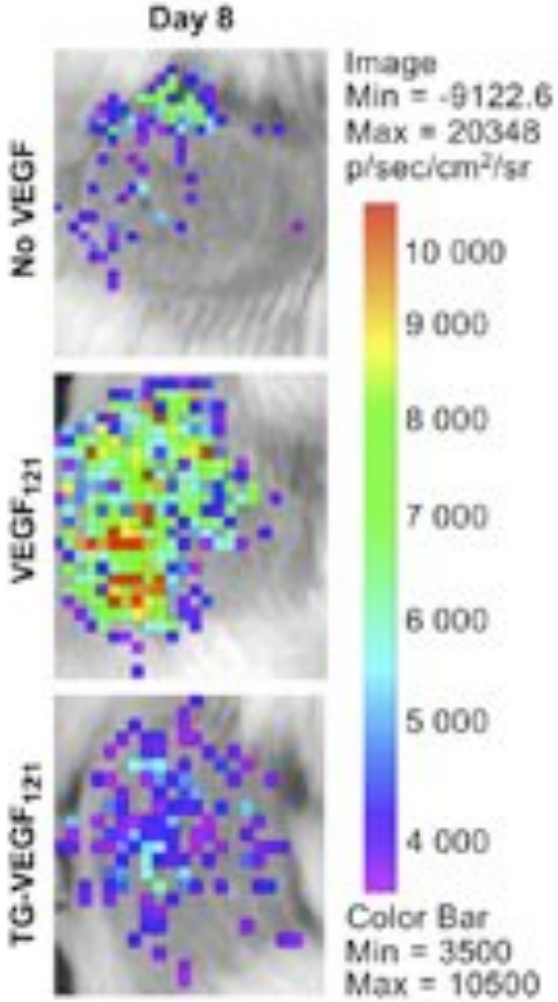


Image from:
M.J. Miller, et al.
PNAS **100**:2604
(2003)

Non-invasive imaging

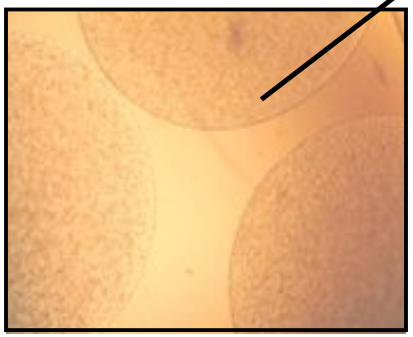
- MRI, tomography, ultraound, other techniques adapted from medical diagnostics
  - can be used to study gene expression
  - requires engineering reporter for the gene
  - can do whole-body optical modality with fluorescence or bioluminescence
- Example: monitoring angiogenesis
  - VEGFR2-*luc* (luciferase reporter)
  - slow- & fast-release VEGF in fibrin scaffolds
  - mice injected with luciferin (substrate) and observed for VEGF receptor upregulation
- Other uses? (think tumors)

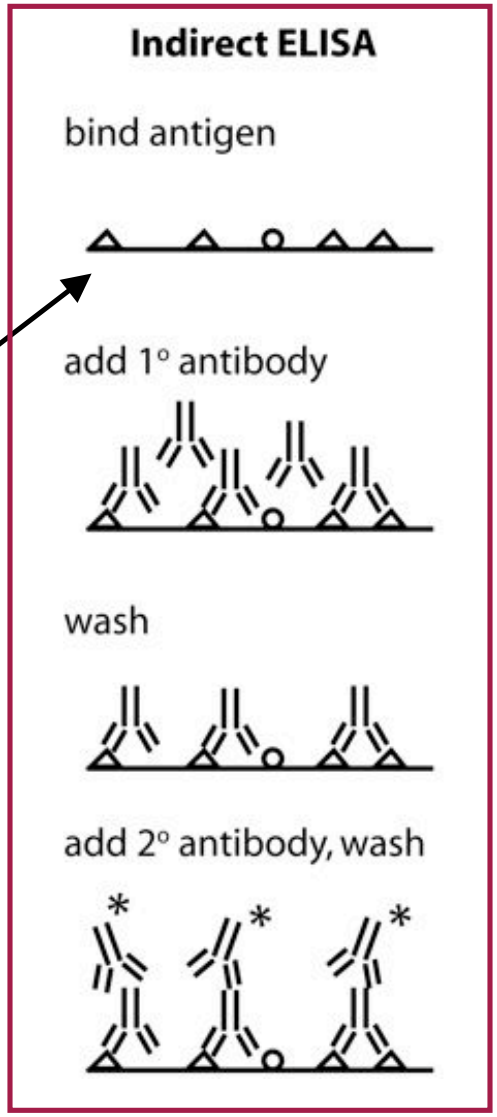


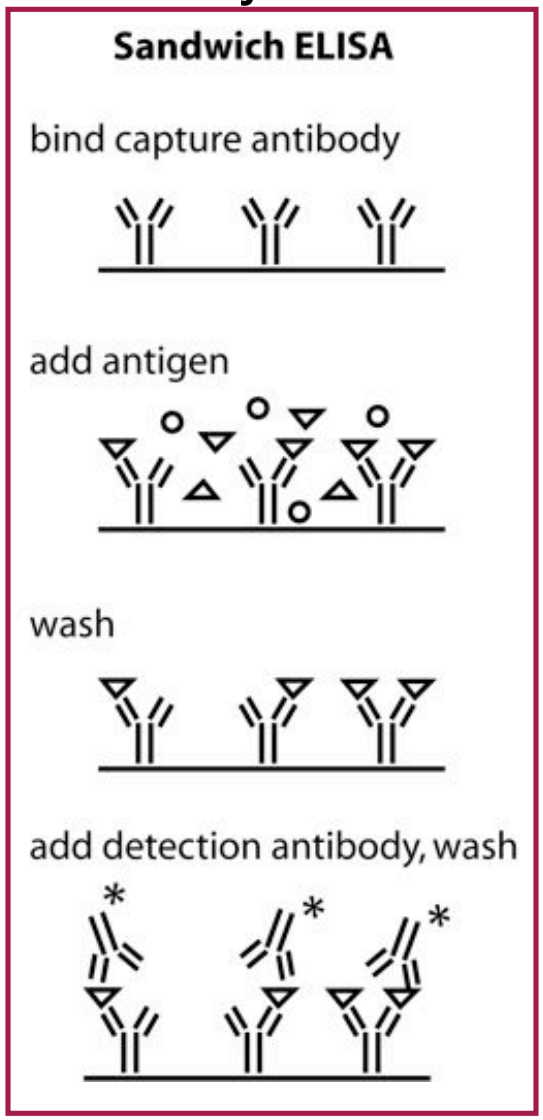
M. Ehrbar, et al. *Biomaterials* **29**:1720 (2008)
Nature News Feature **412**:372 (2001)

Day 5: protein analysis

- ELISA: enzyme-linked immunosorbent assay
  - 96-well format
  - sensitive
  - specific
  - multiple kinds
  - use std. curve

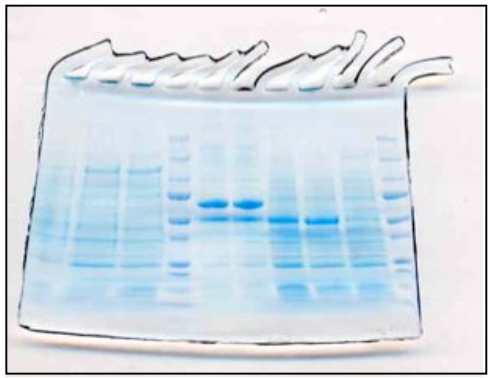


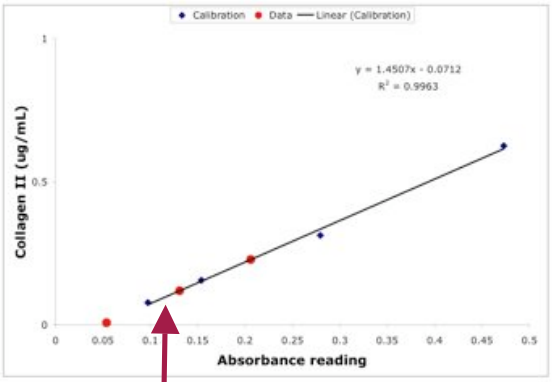




Protein-level assays

- PAGE
  - advantage: relatively straightforward
  - detection limit of 0.3-1 ug/band for Coomassie, 2-5 ng/band for silver
  - cannot distinguish two proteins of same MW
- Western blot
  - advantages: can distinguish specific proteins
  - detection limit ~1 pg (chemiluminescent)
  - only straightforward for denatured proteins
- ELISA
  - advantages: detects native state proteins, quantitative, high throughput, allows competition assays
- Also immunoprecipitation, histology, etc.

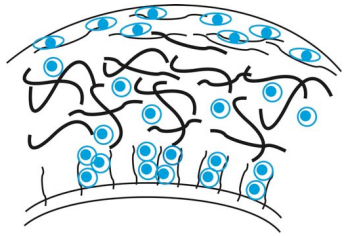




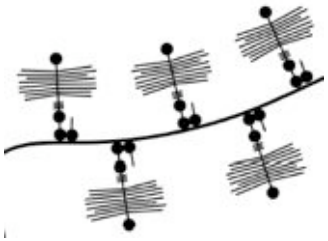
100 ng/mL protein

Current Protocols in Cell Biology, Molecular Biology

Specialty assays for cartilage TE

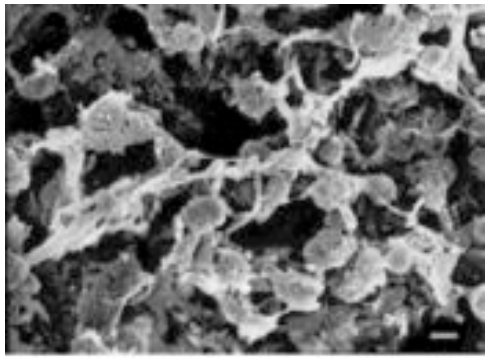


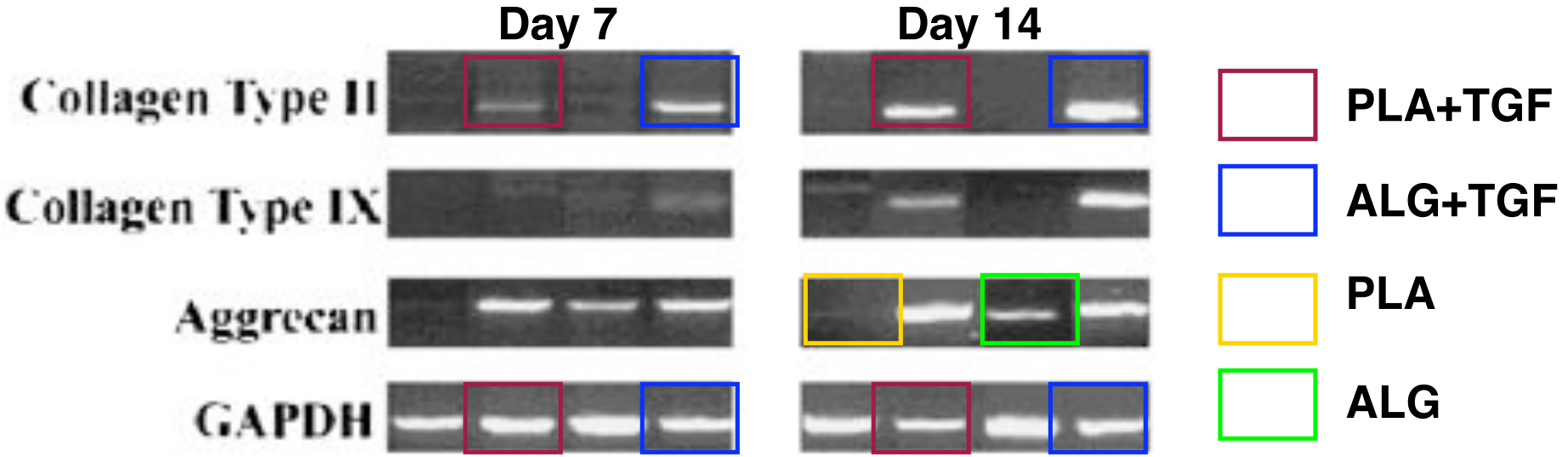
- Gene expression: collagen, aggrecan, cytokines
- Hydroxyproline content - why?
- Collagen can be assessed in different compartments
  - supernatant, cell-associated matrix,and further-removed matrix
    - Ragan et al., Archive Biochem Biophys 383:256, 2000
  - acid or enzyme-soluble collagens
- Assessing proteoglycan content
  - precipitation of PG in supernatant by cetylpyridinium chloride
  - Western blots for different aggrecan domains/componenets
  - measuring PG average size, turnover
  - DMMB (dimethyl-methylene blue) dye - how does it work?
- Mechanical testing
  - test starting tensile or compressive strength of material
  - test for cartilage-like properties after cell growth/ECM secretion



Revisiting *in vitro* cartilage TE

- Porous PLA scaffold + marrow cells
- Cells loaded in alginate vs. medium
  - round morphology, good cell retention
  - alginate alone somewhat chondrogenic
  - alginate+TGF more chondrogenic than PLA+TGF





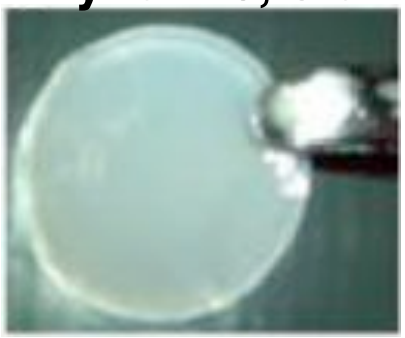
Caterson et al., *J Biomed Mater Res* **57**:394 (2001)

Scaffold-free cartilage TE

- Method: rotational culture of rabbit chondrocytes with no cytokines
- Results
  - Optimal combination of static+dynamic culture promotes stable construct
  - Dynamic cultures had fewer apoptotic cells
  - Microscopy revealed organized architecture
  - Peripheral region similar to *in vivo*
  - New ECM was primarily CN II and PG
- A scaffold-free method is inherently biocompatible
  - what are its disadvantages?
  - what advantages do cell-free methods have?

Static

Dynamic, 3 d



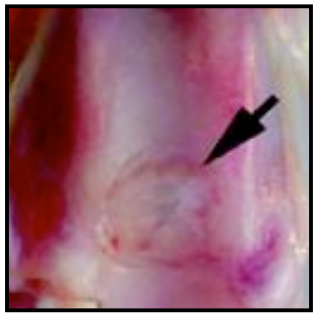
Dynamic, 3 w

T. Nagai et al., *Tissue Eng* **14** (2008)

Example: *in vivo* rabbit model

- Y. Liu et al. *Tissue Eng* **12**:3405 (2006)
- Method: stem cells and/or injectable natural matrix (HA/gelatin) placed in 5-mm knee defects
- Results: matrix promoted greater area of cartilage formation, cells promoted better integration

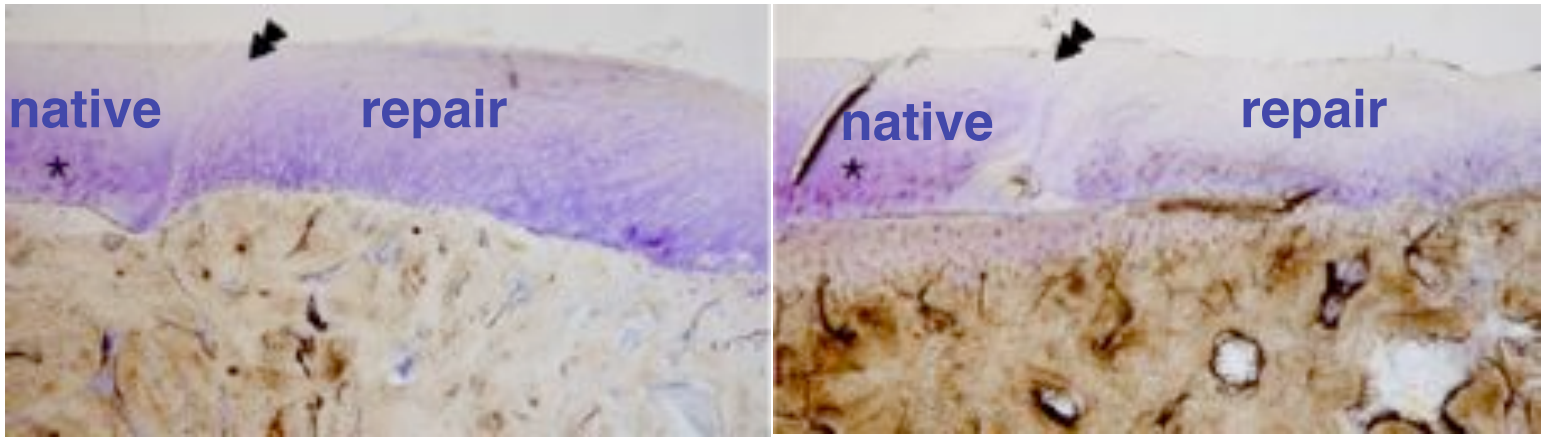
Healing at 12 weeks



			Grade (Points)						
Group	*Interval Until Animals Were Sacrificed (Wks)*	*Specimens* n	*Percentage Defect Filling*	*Articular Surface Continuity*	*Restoration of Osteochondral Architecture*	*Repair Tissue Integration*	*Cellular Morphology*	*Matrix Staining*	*Total Score*
Untreated	4	8	1.25	0.25	0.13	0.25	0.00	0.00	1.88
	8	8	2.20	0.75	0.63	0.50	0.38	0.13	4.59
	12	8	3.12	1.00	1.00	1.13	0.13	0.25	5.63
MSCs only	4	8	1.75	0.38	0.63	0.25	0.38	0.00	3.39
	8	8	3.00	1.38	1.50	1.50	0.38	0.25	8.01
	12	8	3.63	1.25	2.13	1.25	1.25	2.13	11.64
sECM only	4	8	3.63	1.75	3.00	0.50	1.13	0.88	10.89
	8	8	3.63	2.00	3.25	0.50	1.25	2.13	12.76
	12	8	3.75	2.75	3.75	2.75	1.38	2.75	17.13
MSCs + sECM	4	8	3.75	2.50	3.25	1.50	2.00	2.38	15.38
	8	8	3.88	2.75	3.50	2.25	3.63	2.63	18.64
	12	8	4.00	3.00	4.00	3.00	4.38	3.00	21.38

Example: *in vivo* horse model

- D. Barnewitz et al. *Biomaterials* **27**:2882 (2006)
- Method: biodegradable scaffold with autologous cells secured in full-thickness (8 mm) defect in horse
- Results
  - examined horses and dissected joints after 6-12 months
  - collagen and GAG amounts similar in repair and native tissue
  - histology and MRI showed implant integration, ECM formation
- What's new information (vs. *in vitro*), and what's missing?



Advantages of working *in vivo*

- Ability to mimic human disease-state
- Ability to mimic therapy/surgery applied to humans
  - especially true for large animal models
- Can compare results to “gold standard” treatment
- The construct interfaces with an actual wound, the immune system, etc. - more realistic environment
- Toxicity studies more meaningful

Cartilage pathology

- Cartilage has little regeneration capacity
- Acute damage early in life can promote later degeneration, e.g., osteoarthritis
- Osteoarthritis pathology
  - PG content in tissue goes down
  - PG and collagen degrade
  - higher water content
  - resulting reduction in strength
  - chondrocyte death
- Symptoms
  - pain, loss of movement ability

V.C. Mow, A. Ratcliffe, and S.LY. Woo, eds. *Biomechanics of Diarthrodial Joints* (Vol. I) Springer-Verlag New York Inc. 1990

Clinical treatments

- Strategy 1: enhance natural healing capacity
  - cytokine (or other biologic) administration
    - hyaluronic acid, TGF-B, IGF-1, BMPs
  - purposely damage subchondral bone
    - stimulate bone marrow stem cells
- Strategy 2: replace with fresh cartilage tissue
  - cell and/or scaffold implantation or injection
    - must be appropriately contained or anchored
  - total or partial joint replacement
    - with synthetic materials and/or donated tissue
    - invasive (arthroplasty) or fiber-optic (arthroscopy)
- Other treatments include
  - continuous passive motion
  - electrical stimulation
  - debridement (rid debris)

S.W. O'Driscoll. *J Bone Joint Surg* **80**:1795 (1998)
S. Poitras, et al. *Arth Res Ther* **9**:R126 (2007)

Lecture 5: conclusions

- Imaging technologies are varied and powerful, and like any high-information assay require appropriate analysis.
- A variety of assays can be used *in vitro* and *in vivo* to test the success of cartilage and other TE constructs.
- TE constructs that enhance native cartilage repair (via cytokines) or fresh tissue regeneration (scaffolds and/or cell and/or cytokines, grown *in vitro* or *in vivo*) may both be clinically useful in treating osteoarthritis.

Next time: special topics in TE, Atissa on presenting with a partner