# STAGE-SPECIFIC OCMS TO ANALYZE TERATOGEN-INDUCED LIMB DEFECTS

PETER G ALEXANDER

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# PROJECT 2 – LIMB DEVELOPMENT

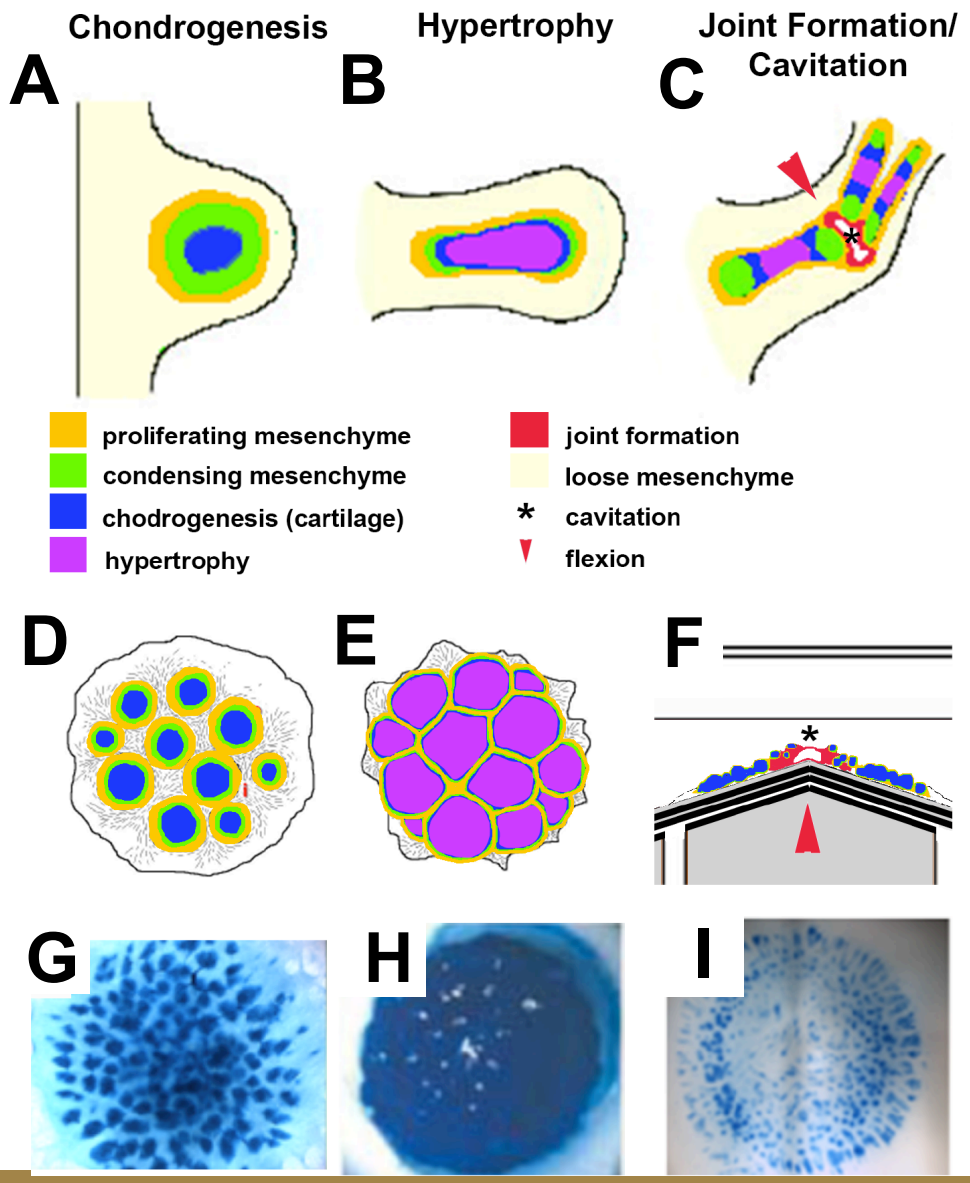
# LIMB MODELS +

## GOALS

**To develop robust in vitro three-dimensional (3D) organotypic culture models (OCMs) based on human mesenchymal stem cells (MSCs) to first examine critical phenomena of embryonic limb development that are prime targets of limb teratogenesis, and then examine their susceptibility to perturbation by known and candidate teratogens and environmental toxicants**

# LIMB MODELS

## SUMMARY OF PROPOSED SYSTEMS TO MODEL SKELETAL DEVELOPMENT IN THE LIMB




5/18/17

# LIMB MODELS

## EXPERIMENTAL MODELING

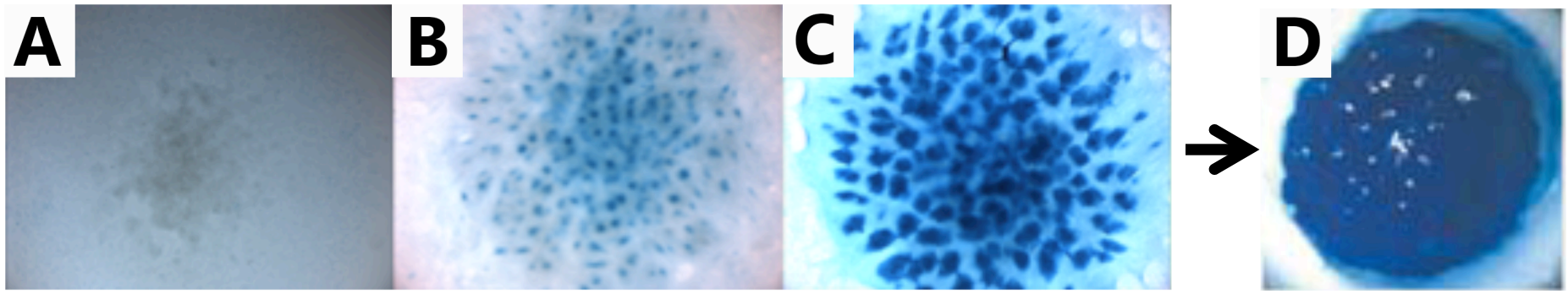
Mesenchymal stem cells (MSCs) derived from adult human bone marrow are used as an analogue cell type for human embryonic limb bud cells and placed in high-density 3D cultures.

These cultures will develop in a manner mimicking (1) cartilage development, and (2) cartilage maturation (hypertrophy)

**Models using embryonic chick limb mesenchyme**

**Cartilage formation**

**Maturation/ Hypertrophy**




Culture Day 1 Day 3 Day 7 Day 21

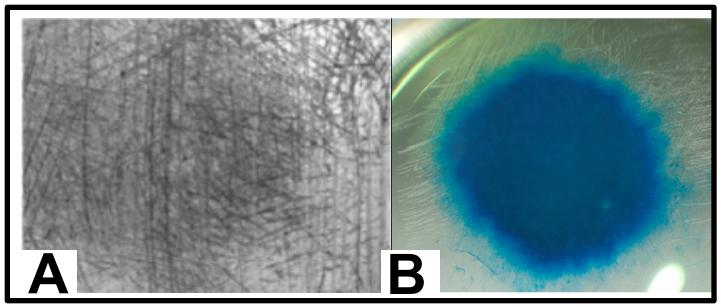
= cartilage nodules stained with Alcian blue to reveal cartilage matrix

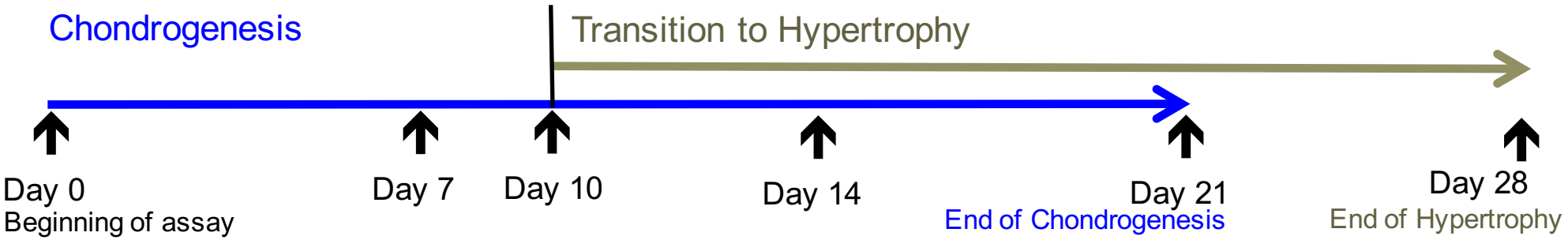
# LIMB MODELS

## CURRENT FORM OF CHONDROGENIC AND HYPERTROPHIC MODELS

**Chondrogenesis:** 2 µL droplet of 20 x $10^6$ cells/ml (40,000 cells) on collagen I-coated texturized tissue culture plastic (48 well plate) with 250 µl of serum-free medium supplemented with TGFβ3 that is changed daily

**Hypertrophy (OCM2):** A chondrogenic planar micromass is cultured in 1 nM dexamethasone + 20 nM thyroid hormone (T3).

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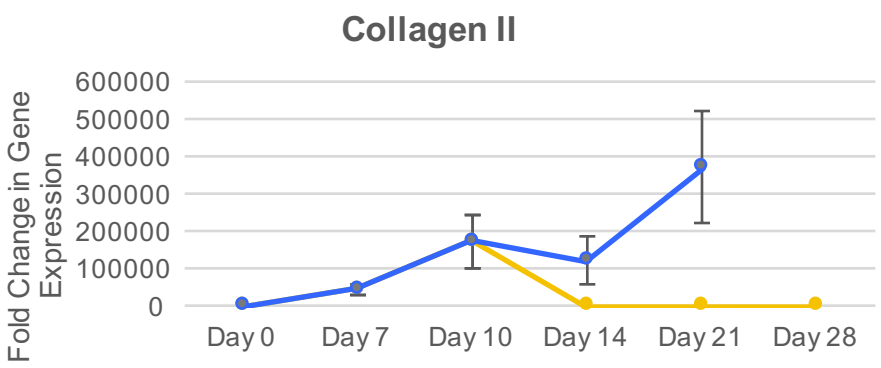
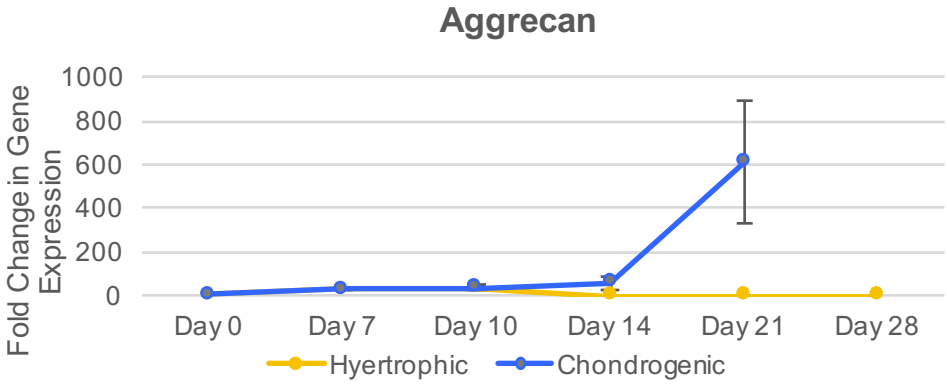
# LIMB DEVELOPMENT

## KEY GENE EXPRESSION ACTIVITIES IN CHONDROGENIC AND HYPERTROPHIC CULTURES

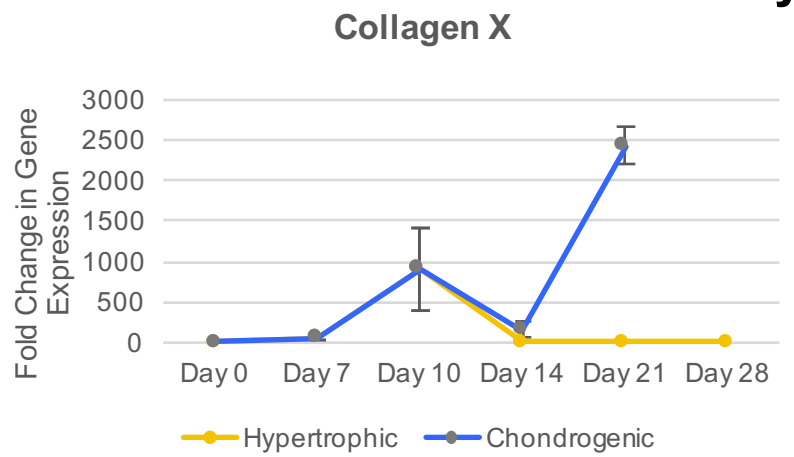
### Chondrogenesis

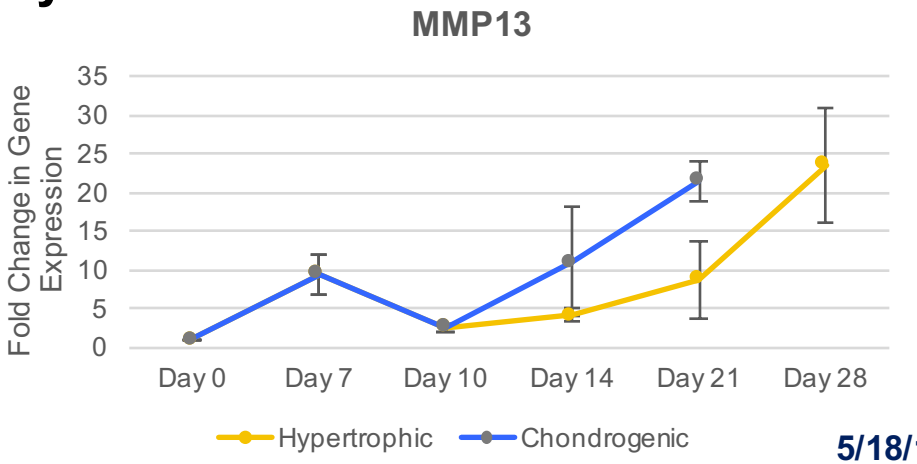



**Chondrogenesis conditions** **Hypertrophy conditions**

### Hypertrophy

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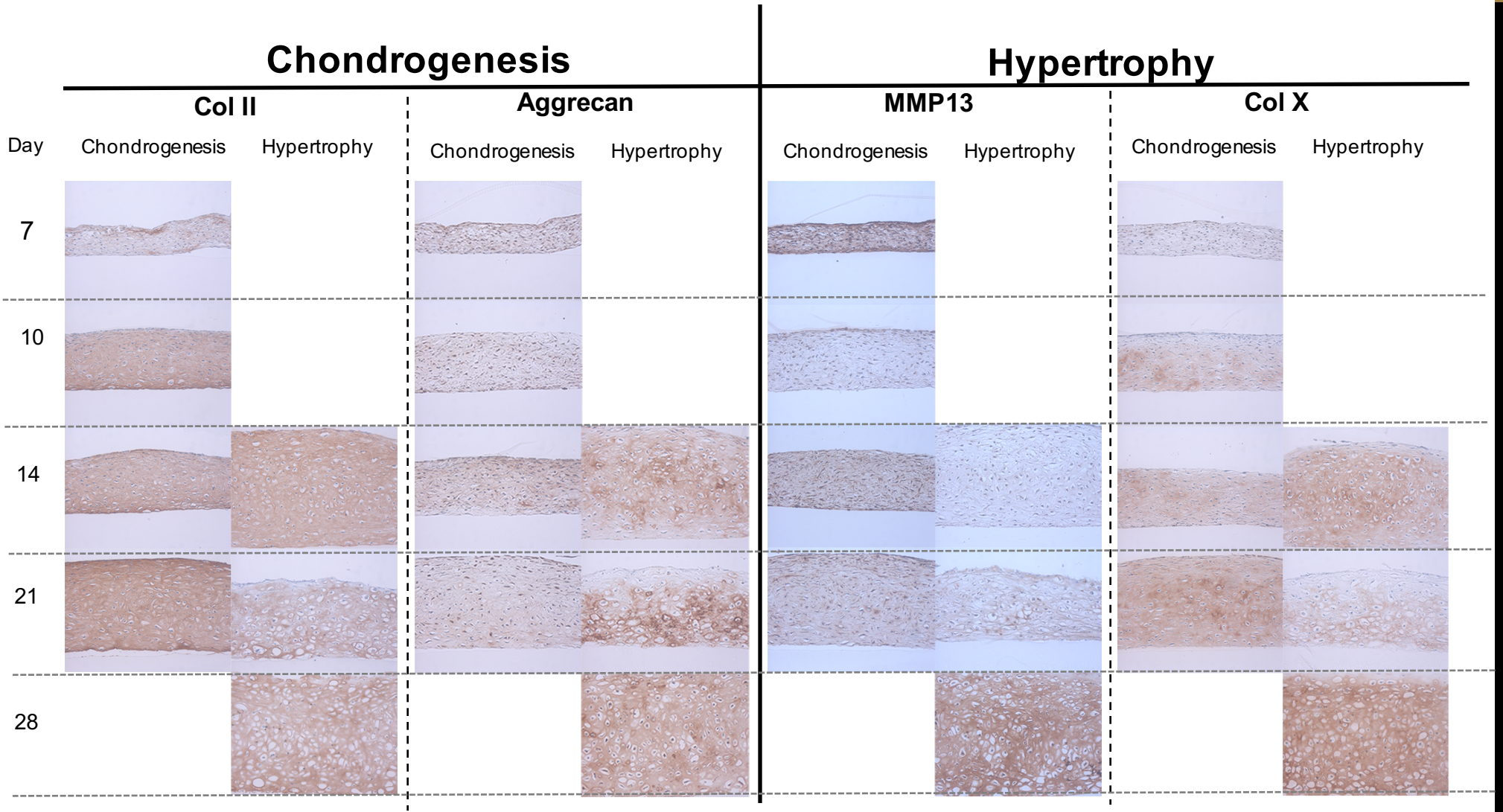
# LIMB MODELS

## EXTRACELLULAR MATRIX ELABORATION IN CHONDROGENIC AND HYPERTROPHIC CULTURES (IMMUNOHISTOCHEMISTRY)

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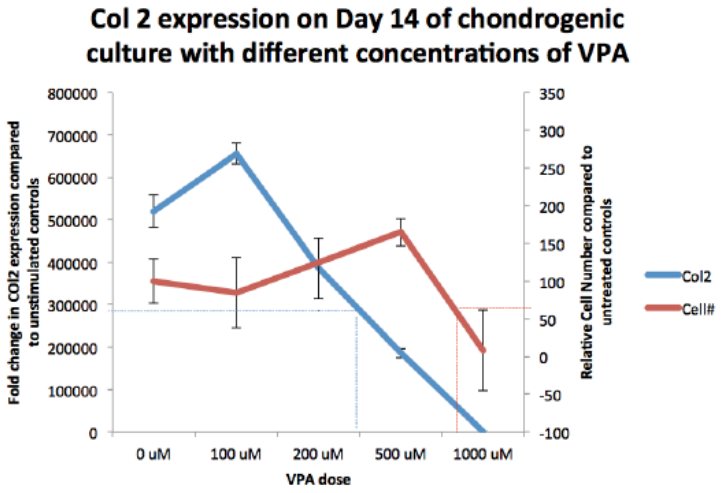
# LIMB MODELS

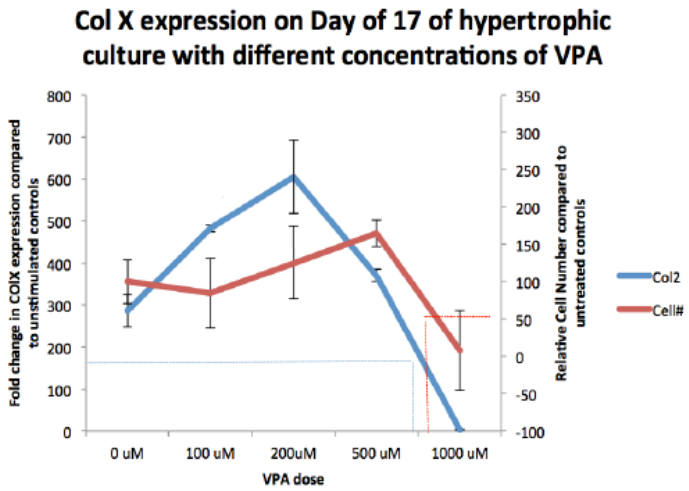
## KNOWN TERATOGENS TO BE TESTED

| Toxicant/toxicant candidates | Medication uses & other uses | Influence on fetuses | Possible mechanism for teratogenesis | plasma [conc.] |
|---|---|---|---|---|
| **Thalidomide** | For alleviation of morning sickness | Severe limb defects incl. Phocomelia | Prevents angiogenic outgrowth during limb formation via inhibition of cereblon-mediated protein turn-over | 2-3 mM |
| **Warfarin** | Anticoagulant in the prevention of thrombosis | Fetal Warfarin syndrome | Inhibits post-translational γ-carboxylation of Glu to form γ-carboxyglutamate(Gla), critical for the action of Gla-containing proteins found in cartilage and bone ECM. | 5-10 uM |
| **Valproic acid** | anticonvulsant and mood-stabilizing drug | Fetal valproate syndrome | inhibition of histone deacetylases (HDACs), which is essential for maintaining the self-renewal and pluripotency of MSCs. (S. Lee et al. 2009) | 0.5 mM |

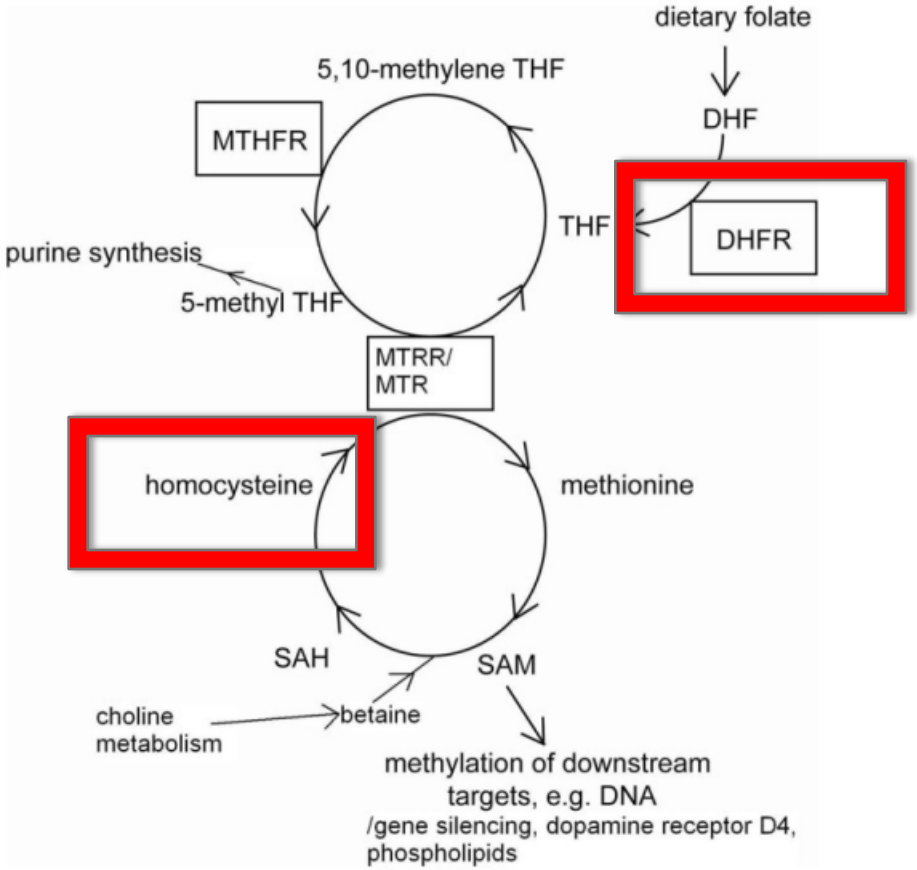
# LIMB MODELS

## EFFECTS OF VPA ON CHONDROGENESIS AND HYPERTROPHY – DOSE RESPONSE




Future correlation of gene expression changes with known AOP



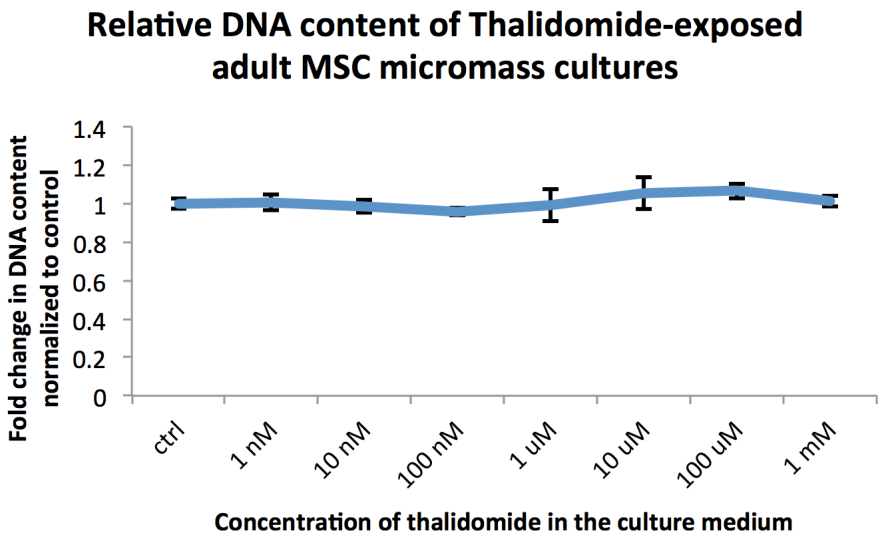

These cultures seem to respond to VPA as we might expect (decrease in differentiation).

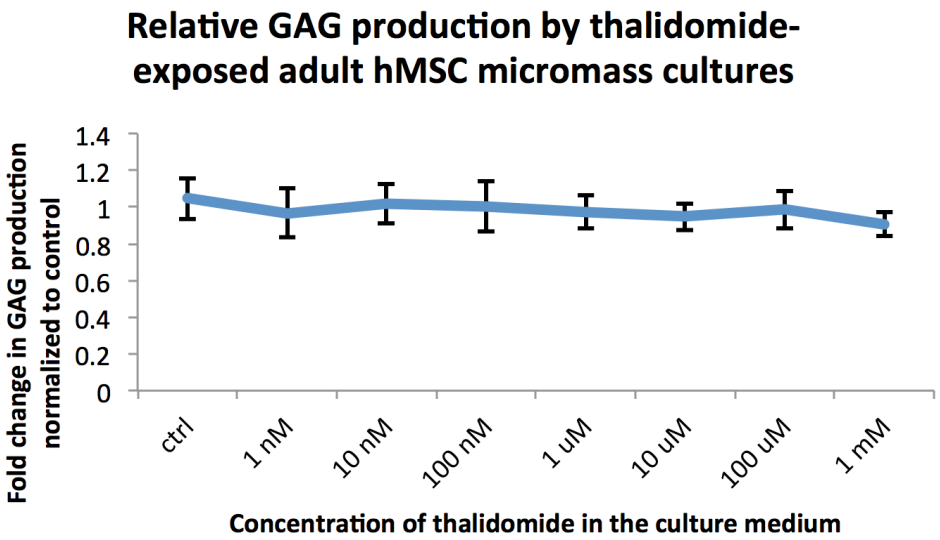
**But this is not true of other toxicants.**

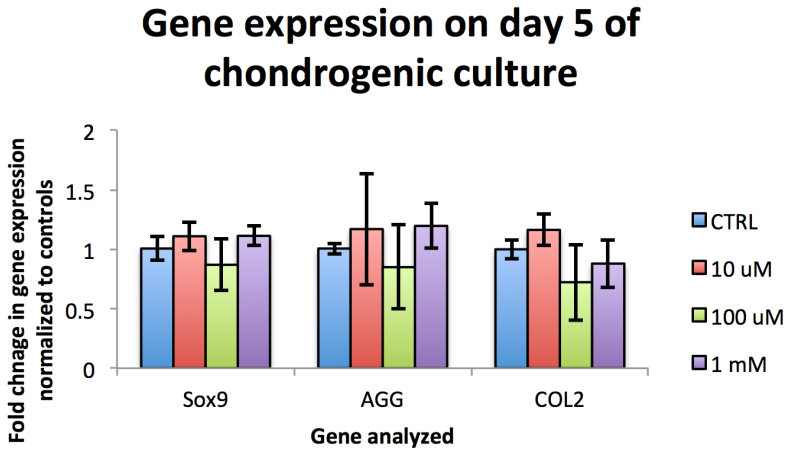
5/18/17

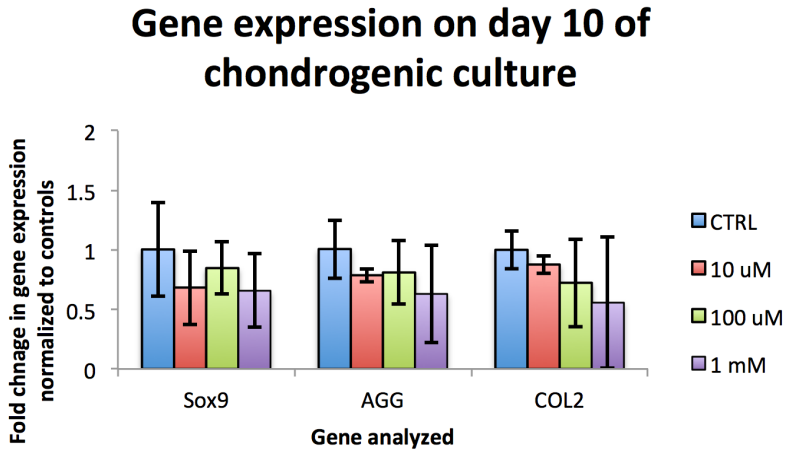
# LIMB MODELS

## EFFECTS OF THALIDOMIDE ON CHONDROGENESIS– DOSE RESPONSE




Thalidomide is such a highly efficient inducer of devastating limb defects,
**this outcome is not satisfactory**

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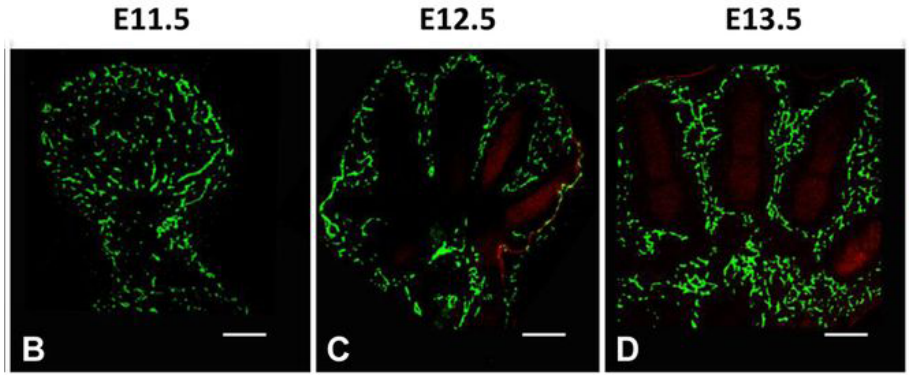
# LIMB MODELS

## ANGIOGENESIS IN LIMB DEVELOPMENT AND PROPOSED MODEL

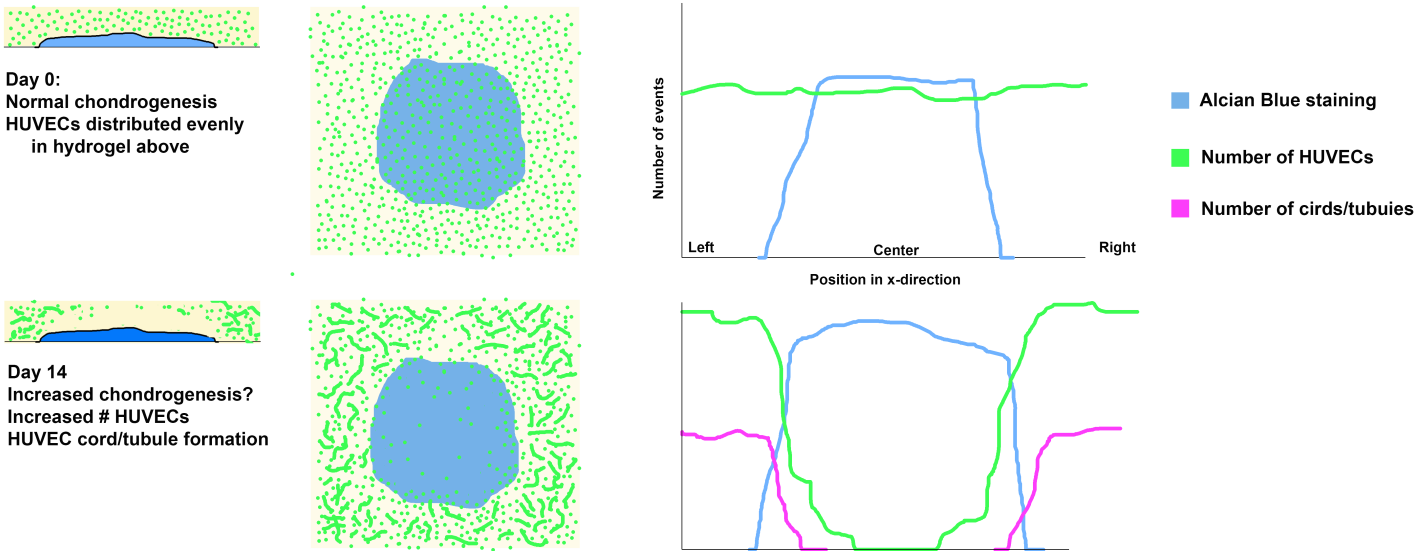
1. Thalidomide may prevent angiogenic outgrowth during limb formation via inhibition of cereblon-mediated protein turn-over

2. Relation of blood vessels and cartilage




Shoham et al. S1P1 inhibits sprouting angiogenesis during vascular development Development 2012 139: 3859-3869.

3. Possible adaptation of micromass to accommodate importance of vasculaogenesis

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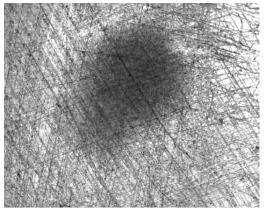
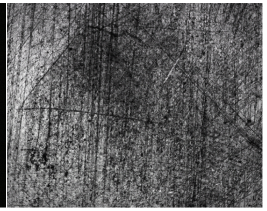
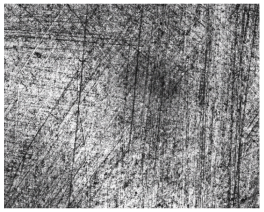
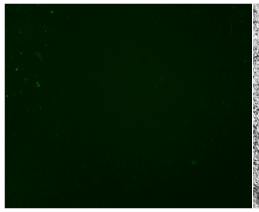
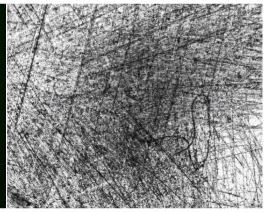
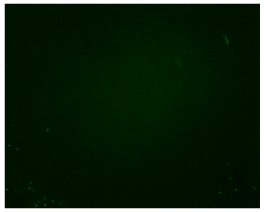
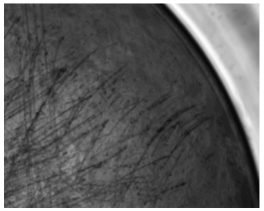
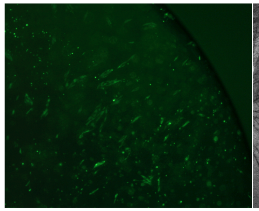
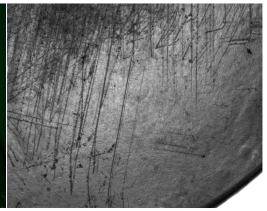
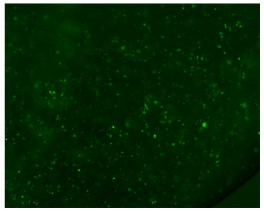
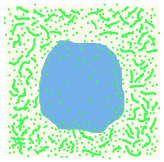
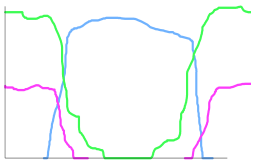
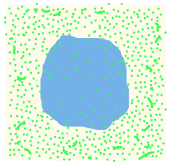
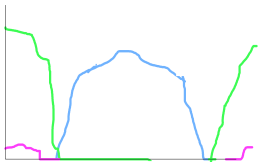
# LIMB MODELS

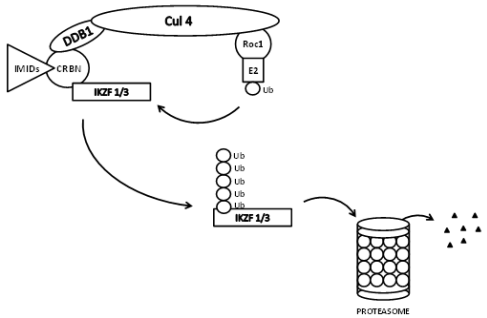
## THALIDOMIDE DISRUPTS HUVEC TUBULOGENESIS

| | Chondrogenic medium | | 1mM Thalidomide | |
|---|---|---|---|---|
| | Bright-field | GFP | Bright-field | GFP |
| MSCs micromass only only |  |  |  |  |
| MSCs micromass and HUVECs overlayed as monolayer |  |  |  |  |
| MSCs micromass and overlying hydrogel-encapsulated HUVECs |  |  |  |  |

**No cords or tubules!**

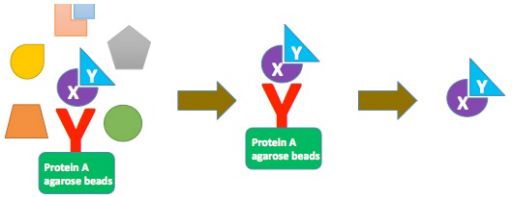





### Future correlation of gene expression changes with known AOP




Fuchs et al. 2014. *Int J Hematol Dis* 1: (2014): 13-20..

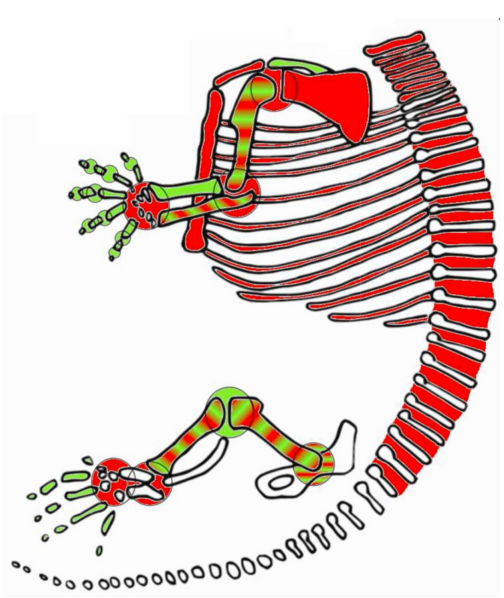
### Co-IP of both chondrocytes and HUVECs



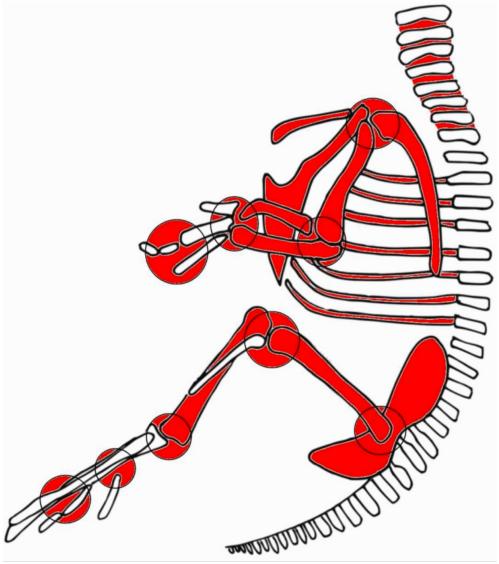

5/18/17

# LIMB MODELS

## PRELIMINARY DATA - CHICK LIMB BUD MESENCHYME



Mouse



Chick

Nowlan et al. 2010. Birth Defects Res C Embryo Today. 2010 Sep; 90(3): 203–213.

We are hypothesizing that mechanical stimulation is necessary for joint tissue differentiation

There is interplay between mechanical forces and skeletal development:

- Mouse mutants non-functional or absent skeletal muscle suffer joint abnormalities
- Pharmaceutical agents which induce paralysis in chick embryos retard joint development

**Defects include:**

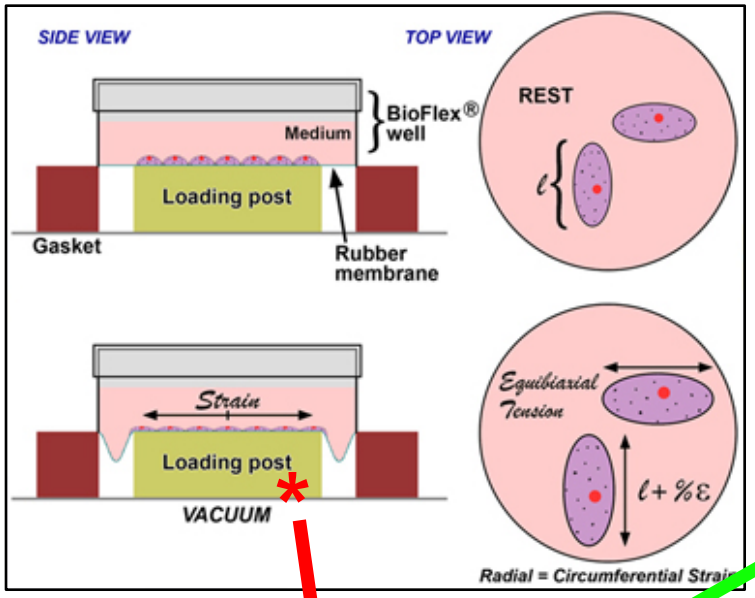
- Fused joints
- Joint surface dysmorphogenesis
- Joint contracture
- Bone, cartilage and/or tendon and ligament involvement

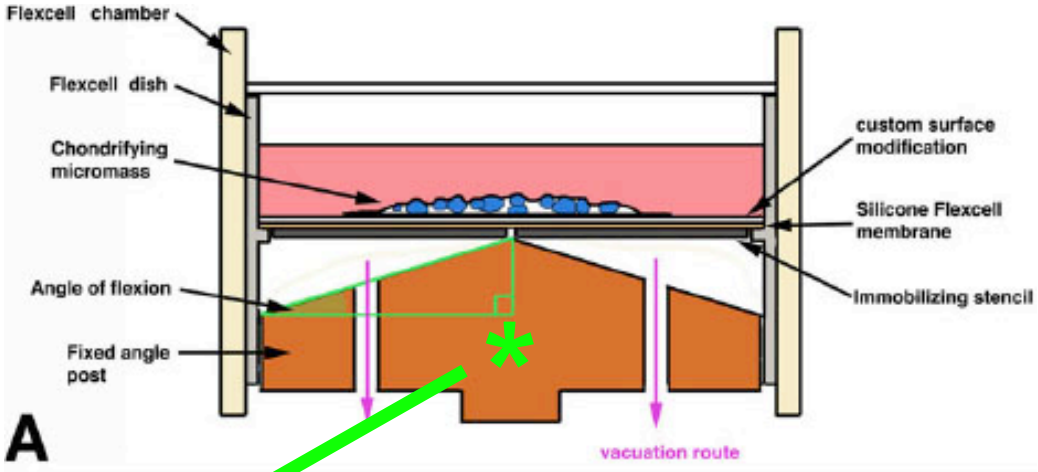
# LIMB DEVELOPMENT

## JOINT SEGMENTATION MODEL:
## PRELIMINARY DATA - CHICK LIMB BUD MESENCHYME

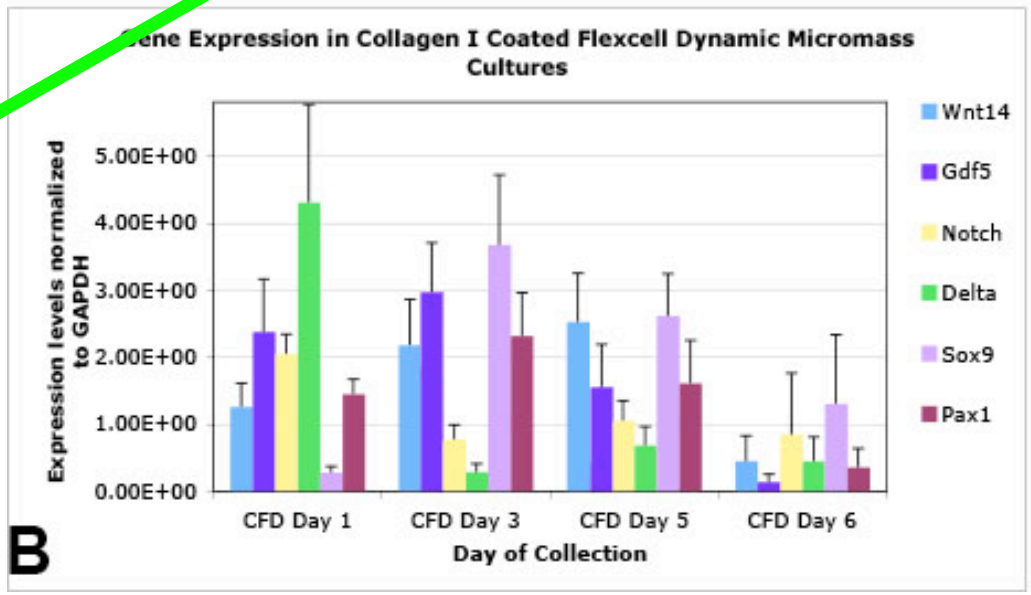
**Hypothesis: *Joint segmentation depends on mechanical activation***

### BioFlex® culture plate



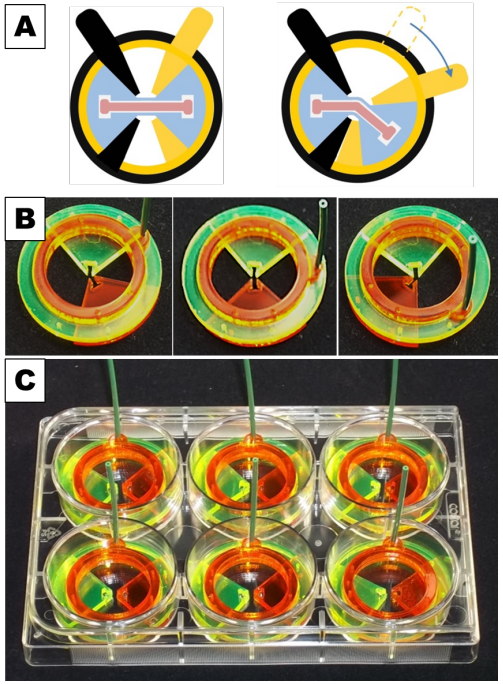

**Change flat loading post to a fixed angle post!**



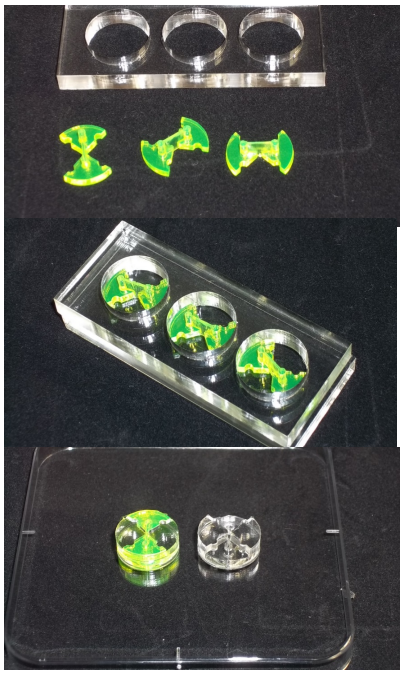
# LIMB MODELS

## JOINT SEGMENTATION MODEL: VERSION 2 FABRICATION (WITH PROJECT 5)

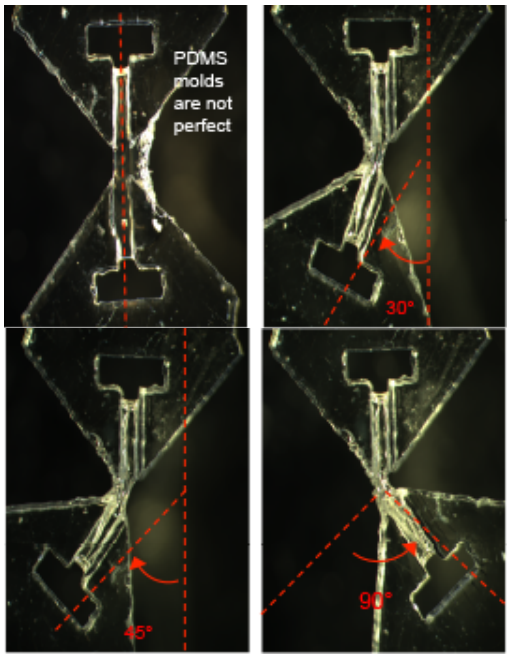
Prototype flexion device has been fabricated




In situ casting system has been developed



A PXL gelatin/HA hydrogel of appropriate density for flexion




**A prototype system is in place to begin mechanically activating a biological specimen**

5/18/17

# LIMB MODELS

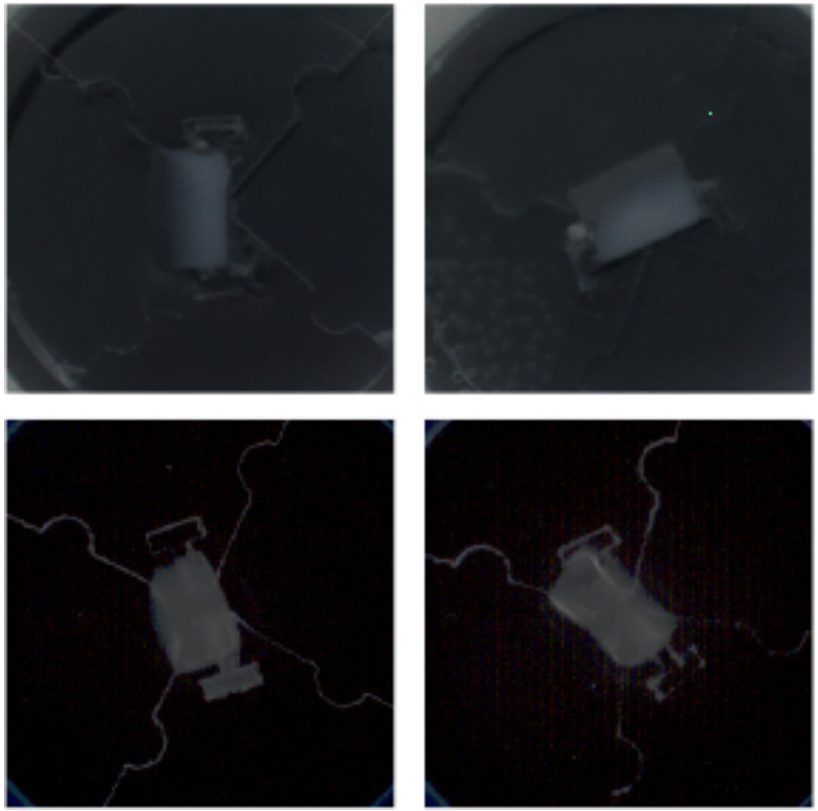
## JOINT SEGMENTATION MODEL
## FLEXION MODEL REVISITED

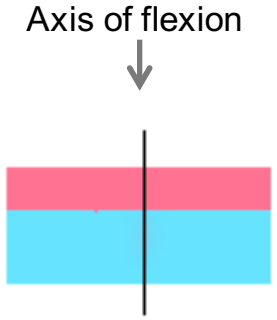
Deliver a construct that can be mechanically stimulated!!
PXL Hydrogel (9% gelatin + 1% hyaluronan, w:v) with 30 x $10^6$ cells/ml

Cast with PDMS casting insert

Day 7

Day 14






**Evidence of joint segmentation:**

- Reduced chondrogenesis
- Joint specific genes

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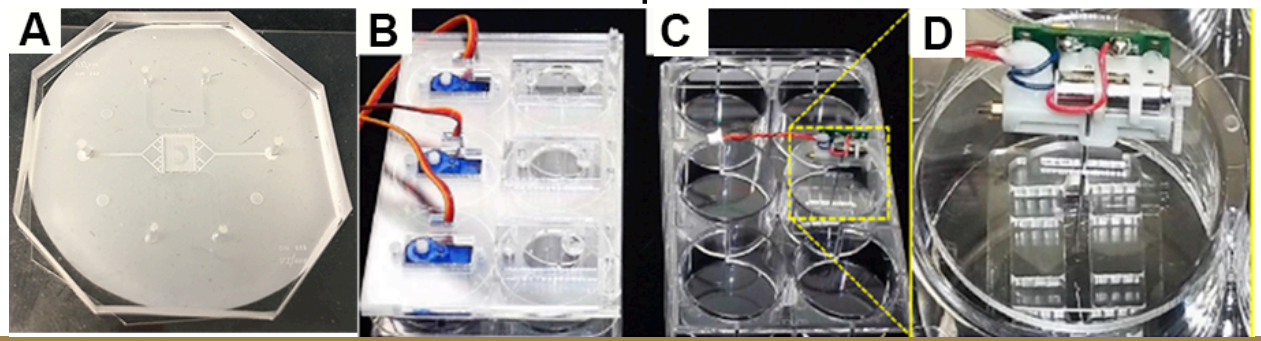
# LIMB MODELS

## Thank You!

….and a hopeful future!

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