The use of zebrafish and mouse models in developmental neurotoxicity, reproductive toxicity and breast cancer risk assessment

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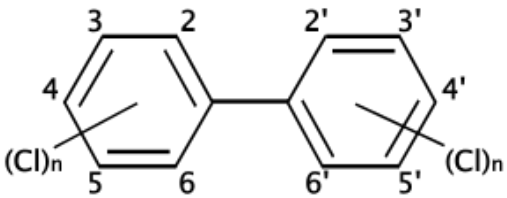
Outline

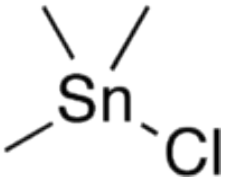
- Zebrafish model
  - Approaches for toxicity mechanism discovery
  - Neurobehavioral toxicity endpoints
  - Reproductive toxicity
  - DNA mismatch repair
- Mouse model
  - Xenoestrogen induced breast cancer risk
  - Aging associated breast cancer risk

Zebrafish Model

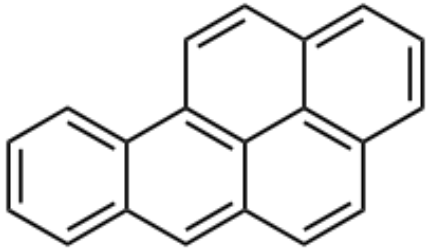
Chemicals



PCBs

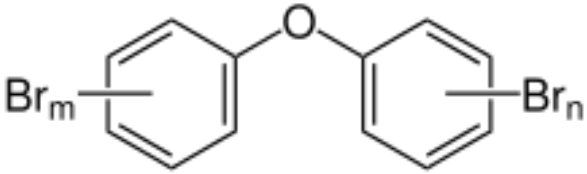


TMT

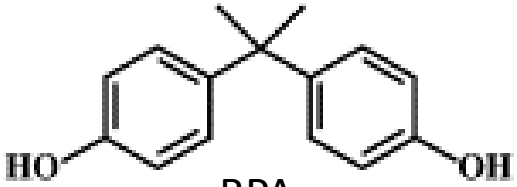


PAHs

PFOS



PBDEs

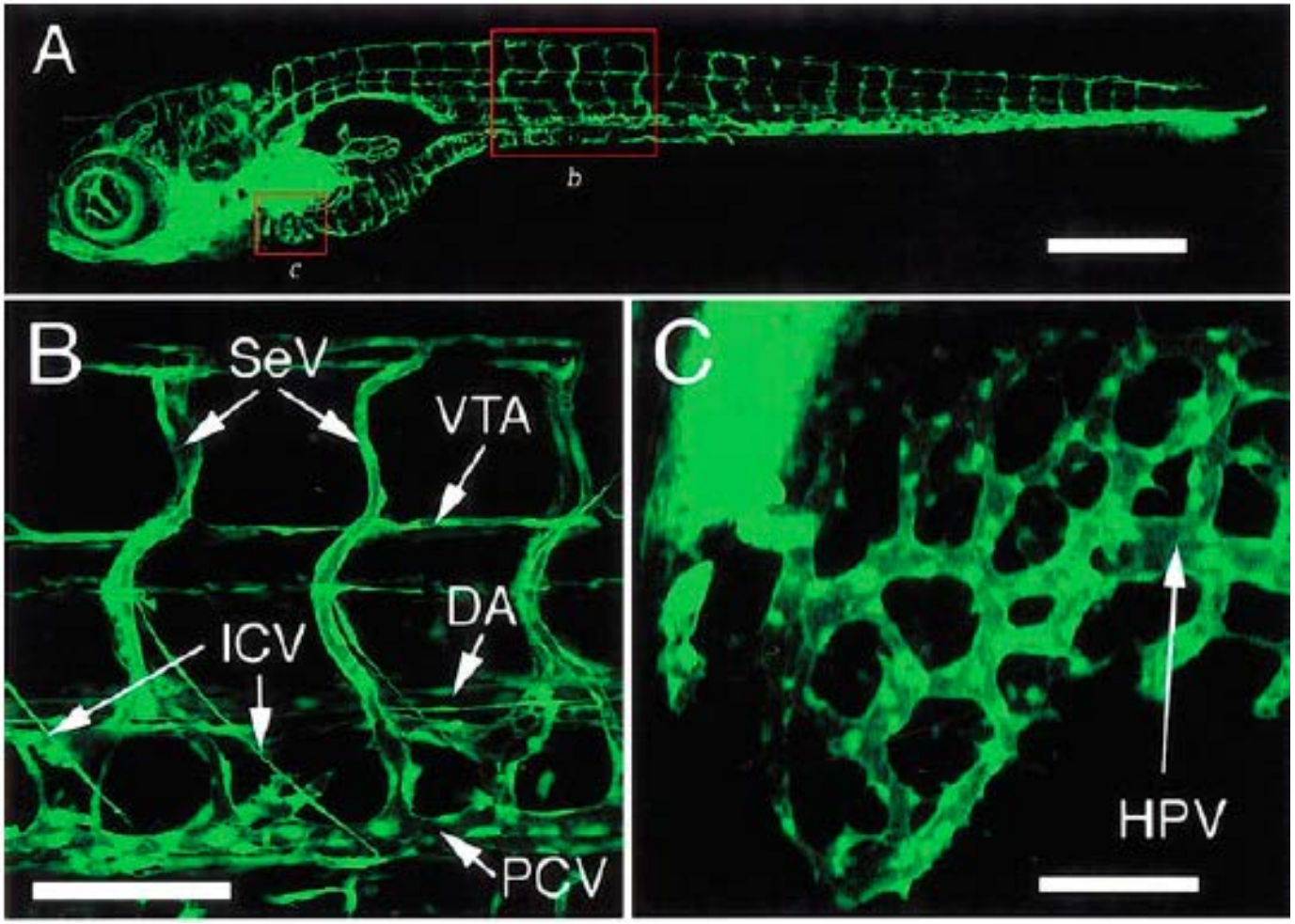


BPA

Trimethyltin Chloride (TMT)

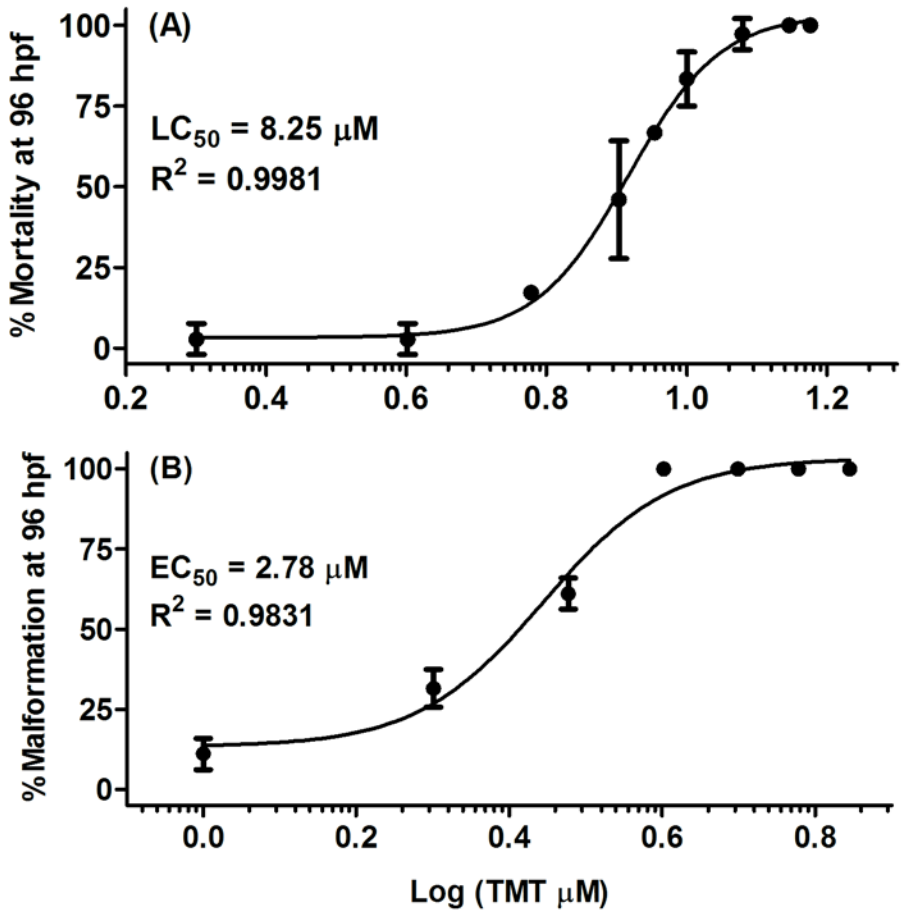
- Organotin
- Polyvinyl chloride, pesticides, fungicides, antifouling paints
- Neuro-, cyto-, immuno-toxicant, apoptotic inducer
- Metal-based chemotherapy for treating human cancers
- Cardiovascular toxicity

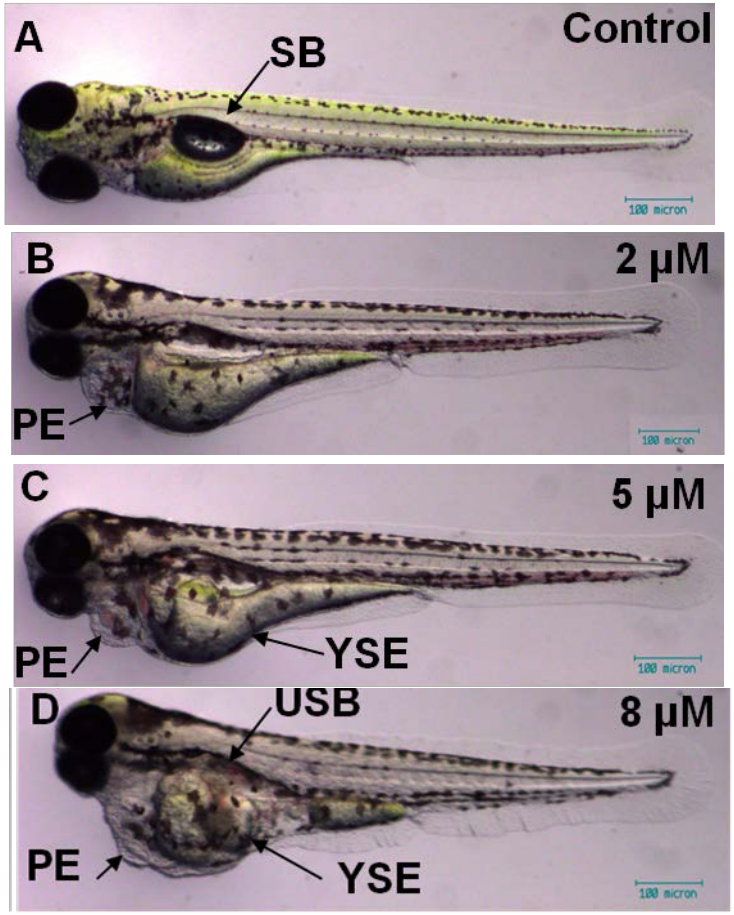
Tg (fli1:EGFP) fish



Lawson and Weinstein, Dev. Biol. 2002

Dose Response Curve

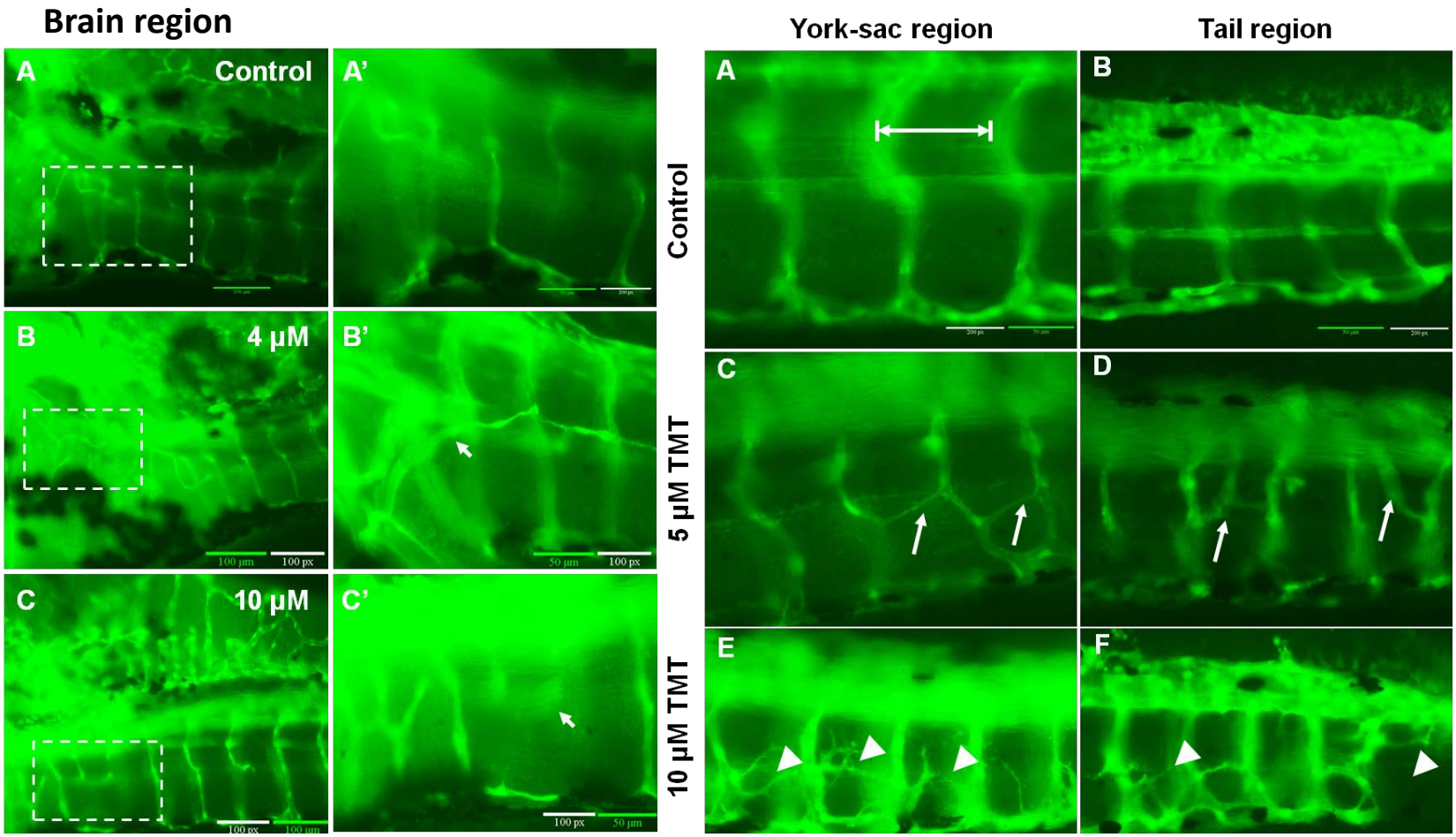




8-96hpf

Chen et al., Toxicology, 2012, 302:129-139

Vasculature Development



Chen et al., Toxicology, 2012, 302:129-139

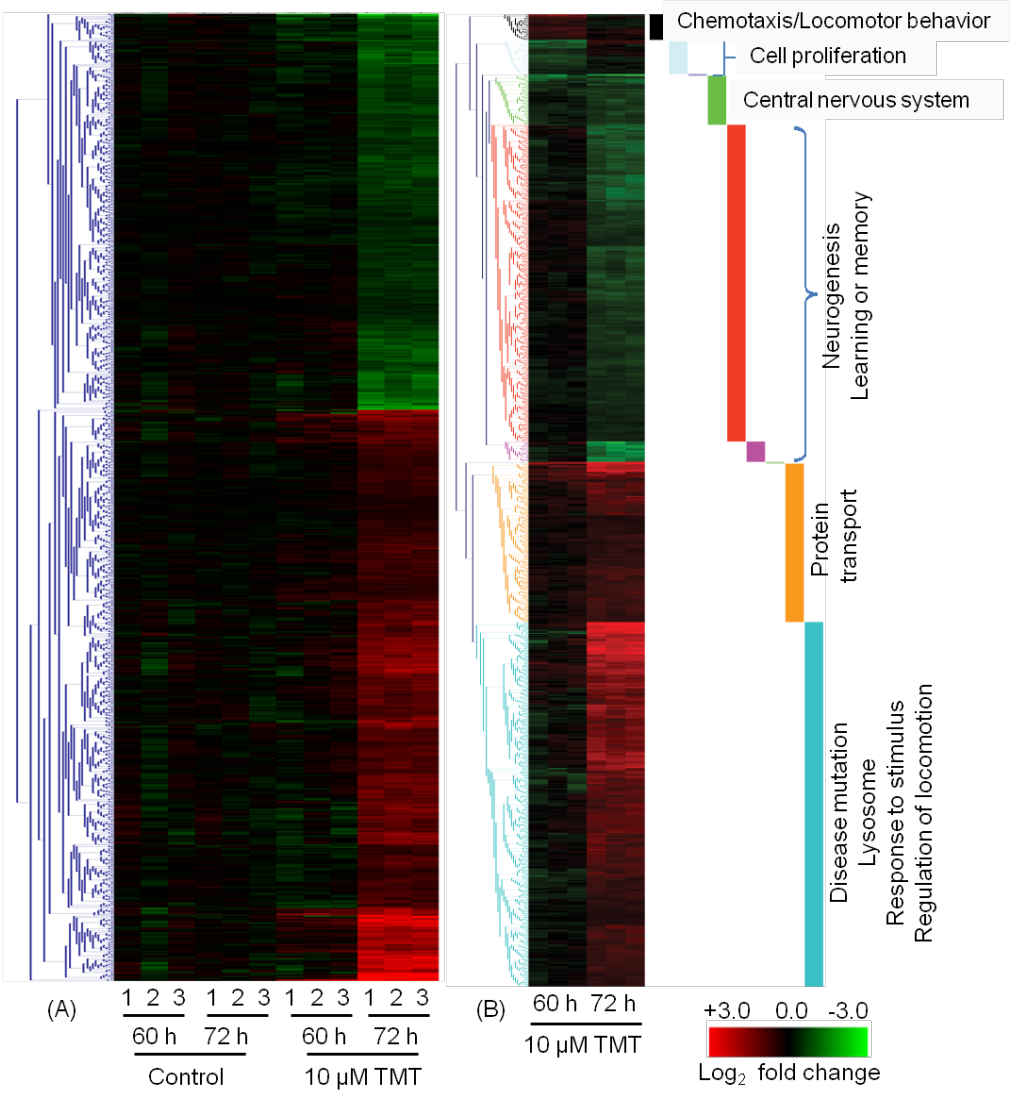
Window of Sensitivity

Table 4
Percent normal morphology, pericardial edema, and mortality of 5D line zebrafish larvae at 96 hpf after TMT exposure at various developmental window ($n = 16$ embryos/larvae per group).

TMT (μM)	Exposure window (hpf)	% Normal larvae	% Pericardial edema	% Mortality
0	8–96	100	0	0
5	8–24	94	6	0
	24–48	94	0	0
	48–72	63	37	0
	72–96	88	12	0
10	8–24	81	19	0
	24–48	19	81	0
	48–72	0	100	0
	72–96	88	13	0
15	8–24	92	8	0
	24–48	0	100	50
	48–72	0	100	87
	72–96	88	12	0

Chen et al., Toxicology, 2012, 302:129-139

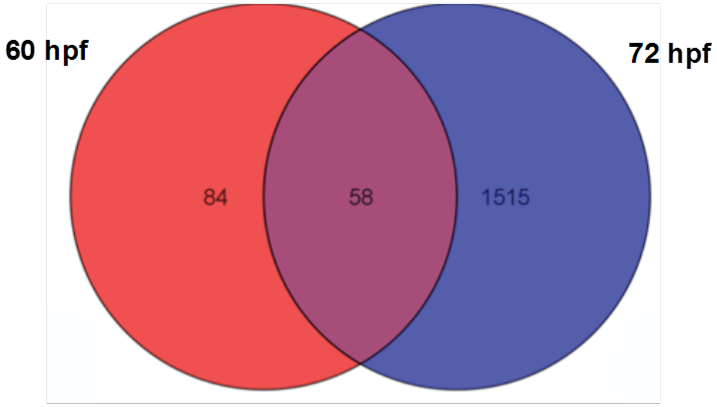
Transcriptional Changes



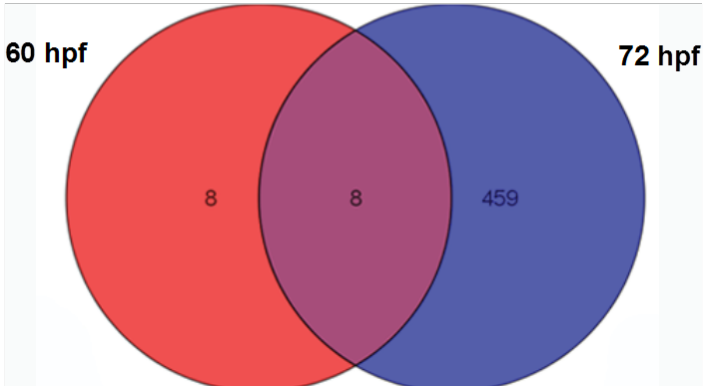
Chen et al., Toxicology, 2012, 302:129-139

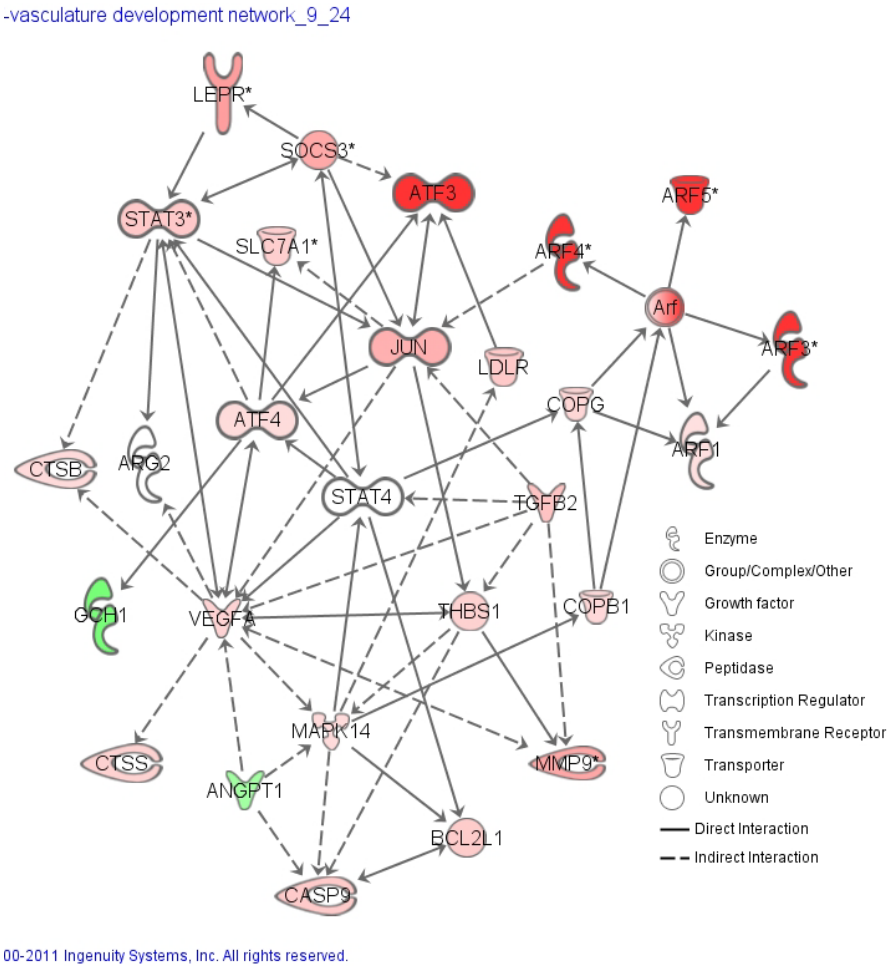
Differentially Expressed Genes

A: Genes with significant changes



B: Genes with > 2 fold changes





Chen et al., Toxicology, 2012, 302:129-139

Microarray vs. qRT-PCR

Table 5
Comparisons of TMT-regulated transcripts in larvae at 72 hpf between microarray and qRT-PCR analysis.

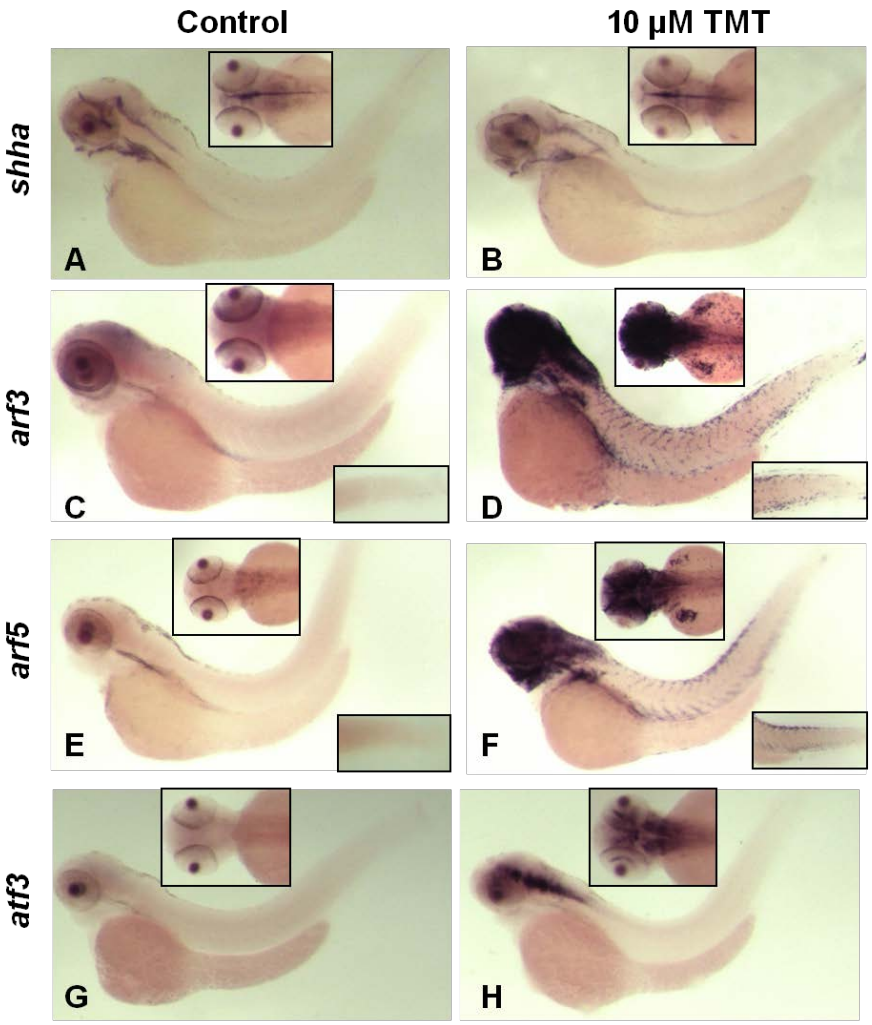
Gene	Microarray			qRT-PCR			Gene targeting effect	SEQ.ID
	Fold	SEM	*P*-value	Fold	SEM	*P*-value		
arf3	30.2	4.4	0.02	1938.5	405.2	0.04	ADP-ribosylation factor related family member	TC252426[a]
arf5	15.1	5.5	0.12	1523.4	174.6	0.01	ADP-ribosylation factor related family member	ENSDART00000056047[b]
atf3	14.4	1.6	0.01	65.3	6.1	0.01	Activating transcription factor 3 (*atf3*)	OTTDART00000006631[c]
zgc:1105152	11.9	0.6	0.00	32.8	6.3	0.04	–	NM_001017853[d]
rfp582	10.7	2.5	0.06	42.0	5.3	0.02	–	ZV700S00001968[e]
stmn4l	9.9	1.2	0.02	14.3	1.4	0.01	Stathmin-like 4: differentiation and proliferation	OTTDART00000025126
Novel	9.4	3.5	0.14	43.9	6.2	0.02	–	ENSDART00000058949
fosl1	9.3	1.8	0.05	81.4	8.0	0.01	Protein similar to vertebrate FOS-like antigen 1	OTTDART00000023370
NID 12	8.6	1.9	0.06	14.6	2.0	0.02	–	ZV700S00006532
NID 14	8.6	3.0	0.12	45.6	8.8	0.04	–	ZV700S00000639
NID 13	8.1	1.4	0.04	12.4	1.9	0.03	–	TC254817
bbc3	7.8	1.9	0.07	12.8	2.1	0.03	Control permeable of the outer mitochondrial membrane	ZV700S00004671
NID 19	7.4	1.5	0.05	16.3	2.6	0.03	–	ZV700S00002527
atf5	7.3	1.1	0.03	15.2	0.9	0.00	Activating transcription factor 5	OTTDART00000021442
Lepr	4.9	1.1	0.07	5.5	0.5	0.01	Leptin receptor; type 2 diabetes mellitus	ENSDART00000104616
mmp9	4.6	1.3	0.11	14.8	2.2	0.02	Matrix metalloproteinase 9	OTTDART00000025258
zgc:162630	4.5	0.5	0.02	41.2	2.8	0.00	–	NM_001082825
fam63a	3.7	0.6	0.04	11.1	0.3	0.00	–	OTTDART00000017116
gcrk7a	−3.4	0.6	0.06	−5.7	0.9	0.04	G-protein-coupled receptor kinase 7a	OTTDART00000024144
Ndellb	3.4	0.5	0.04	4.4	0.1	0.00	NudE-like protein 1b: neurological disorders	OTTDART00000024237
opsin1	−3.3	0.6	0.07	−7.1	0.8	0.02	Opsin 1 (cone pigments), short-wave-sensitive 2	OTTDART00000002229
gnat2	−3.0	0.8	0.13	−6.9	1.1	0.04	G-protein alpha transducing activity polypeptide	ZV700S00006718
arg2	2.8	2.1	0.49	13.8	0.3	0.00	Arginase, type II; smooth muscle disorder	OTTDART00000026242
Galca	2.8	0.9	0.18	2.7	0.6	0.09	Galactosylceramidase: cellular surface cancers	OTTDART00000004460
nnara6	−2.7	0.4	0.06	−4.9	1.2	0.09	Neuronal nicotinic acetylcholine receptor alpha 6	NM_001042684
fen1	−2.6	0.3	0.03	−3.8	0.2	0.01	Elongation of very long chain fatty acids (*fen1*)	BC060897.1[f]
casp9	2.5	0.1	0.00	4.5	0.5	0.02	Apoptosis-related cysteine peptidase	OTTDART00000028081

Note. Fold changes with minus signs indicate down regulation. The sequence IDs were identified using designated online resources.

[a] http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=zfish.
[b] http://www.ensembl.org/index.html.
[c] http://vega.sanger.ac.uk/index.html.
[d] http://www.ncbi.nlm.nih.gov/nuccore.
[e] http://www.ncbi.nlm.nih.gov/nucest.
[f] http://www.ncbi.nlm.nih.gov/gene.

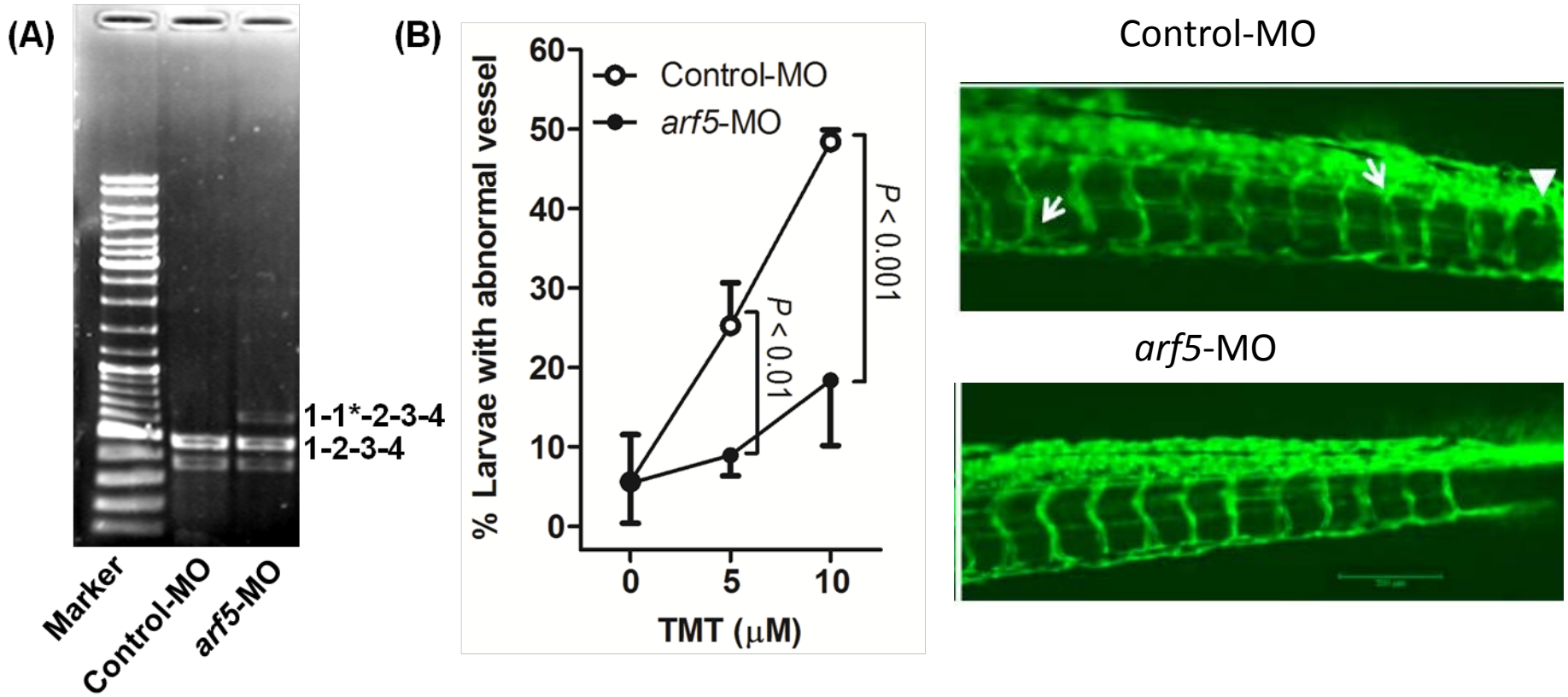
Chen et al., Toxicology, 2012, 302:129-139

Whole Mount in situ Hybridization



Chen et al., Toxicology, 2012, 302:129-139

Morpholino Microinjection

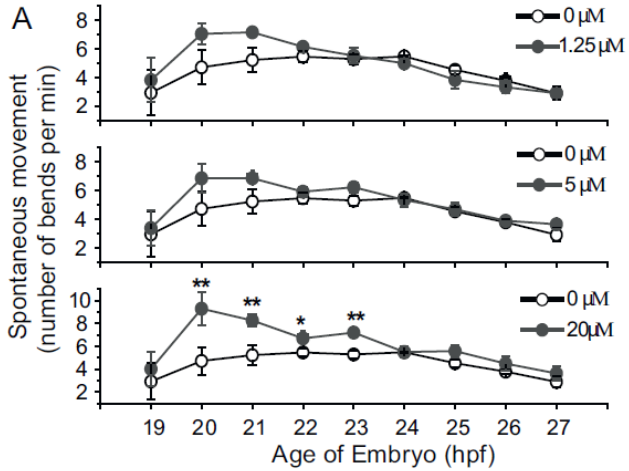


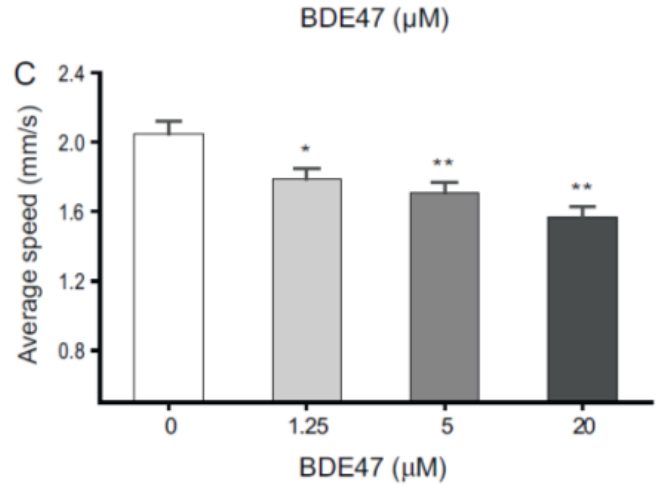
Chen et al., Toxicology, 2012, 302:129-139

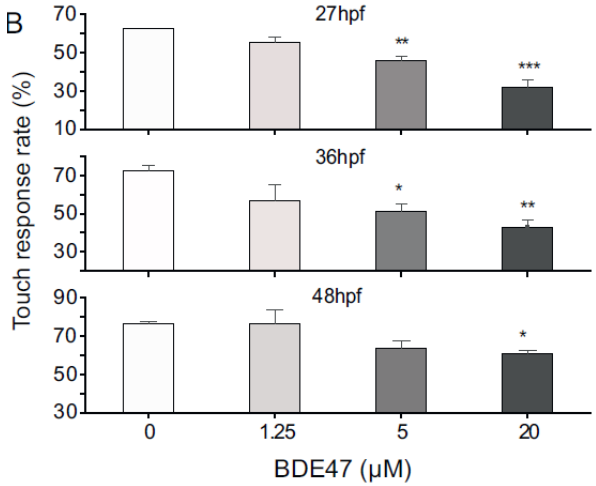
Neurobehavioral Toxicity-Behavioral Tests

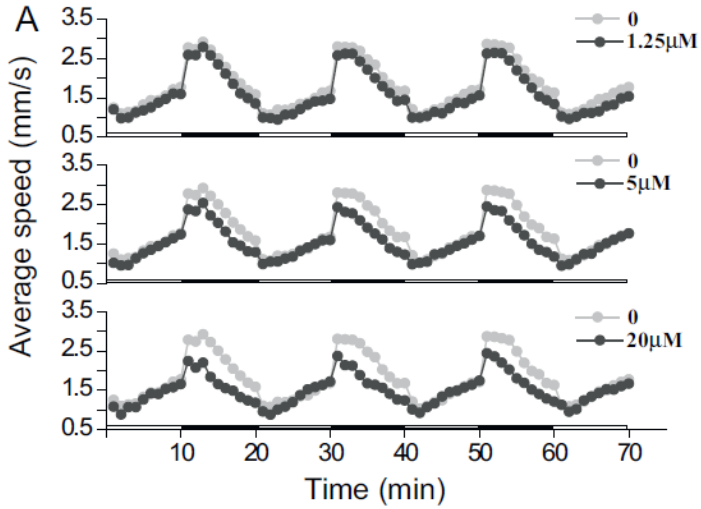
- Spontaneous movement
  - First movement (17-19 hpf)
  - Originates from spinal neuron innervations
- Touch response
  - Escape response (21 hpf)
  - Local spinal motor neurons, targeted muscle fibers, and spinal sensory neurons
- Free swimming
  - Motor neurons and targeted muscle fibers
- Alternating light-dark photoperiod stimulation
  - Visual acuity

Example-BDE47



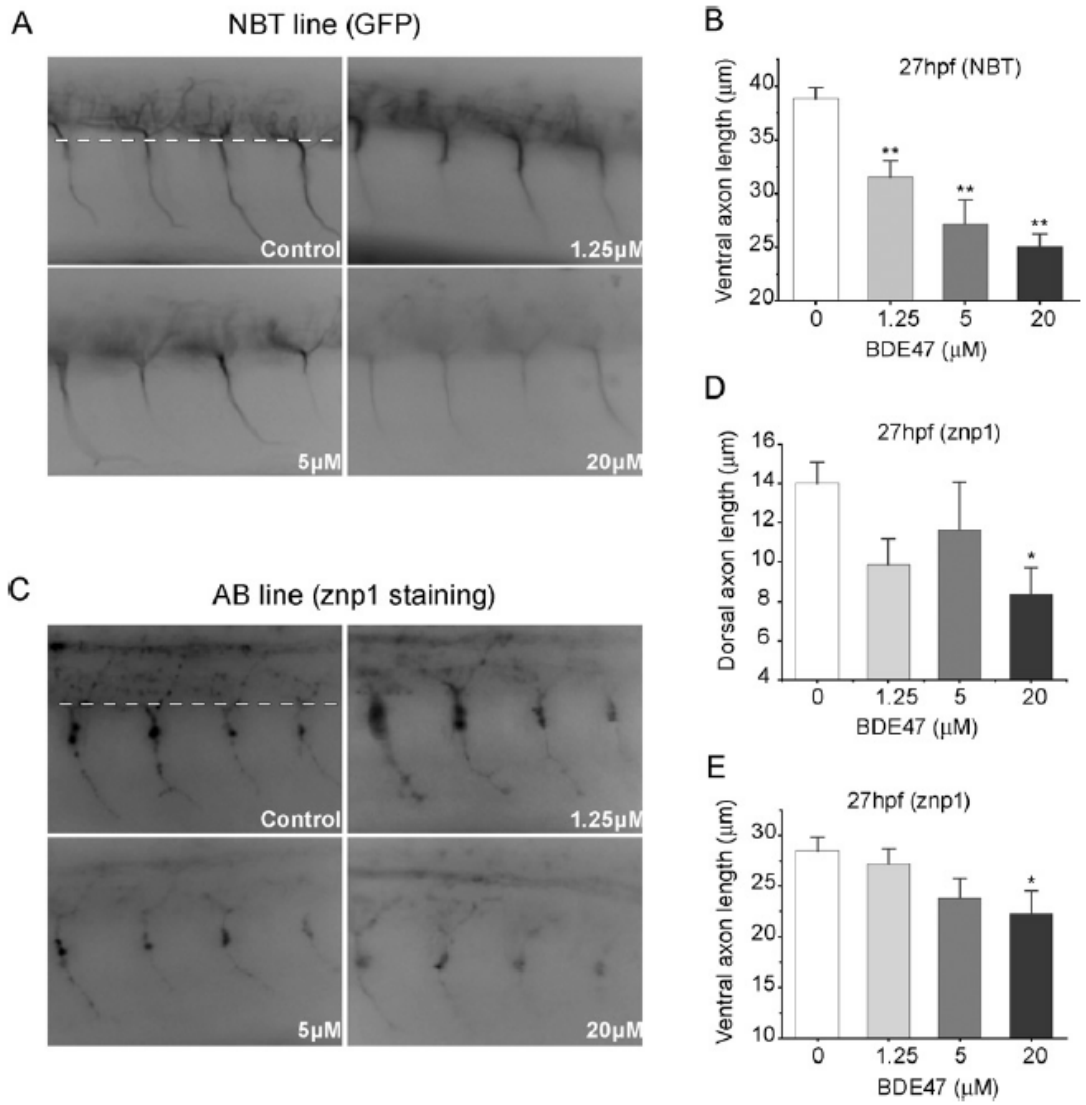




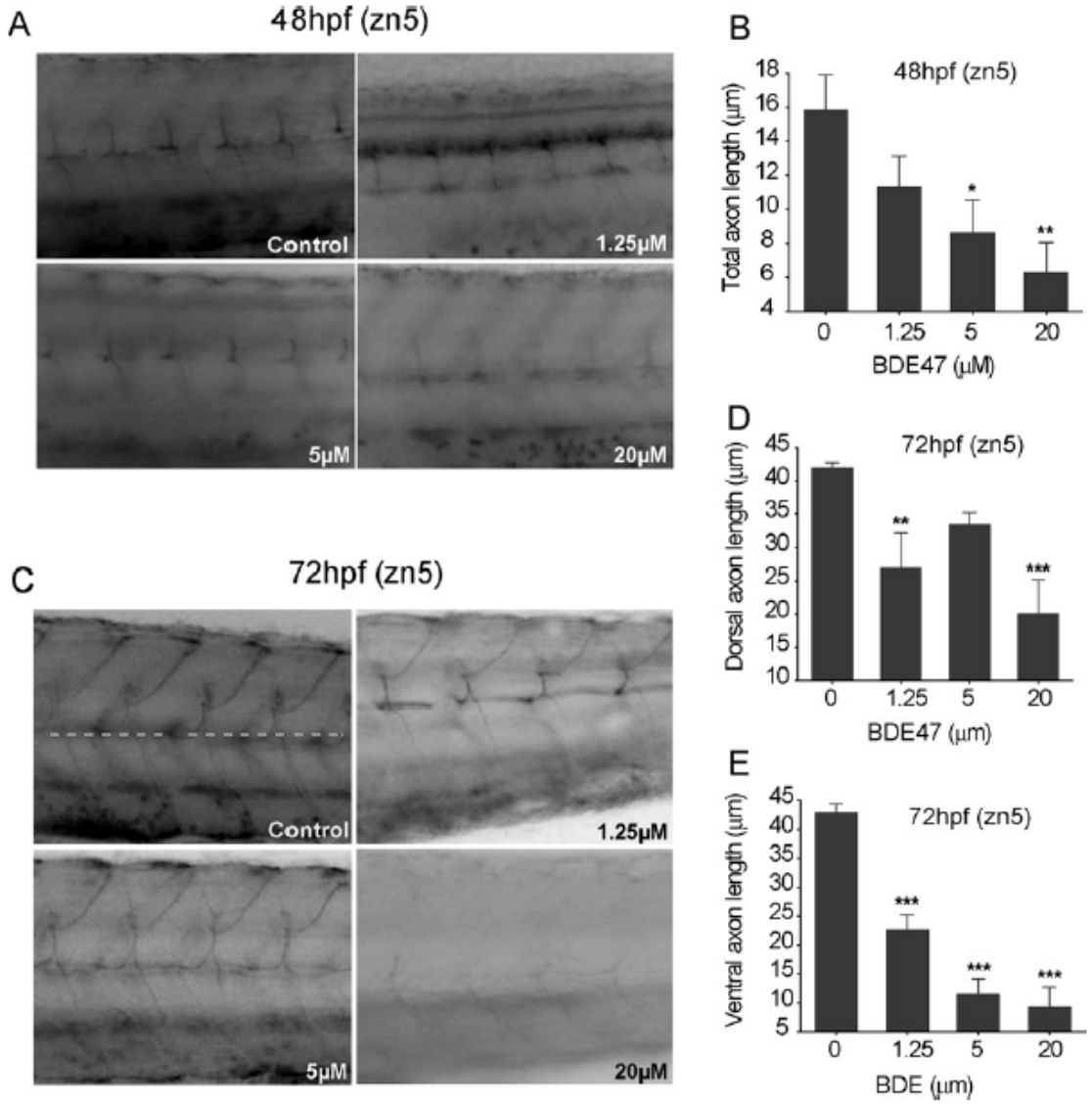


Chen et al., Aquatic Tox, 2012, 120-121:35-44

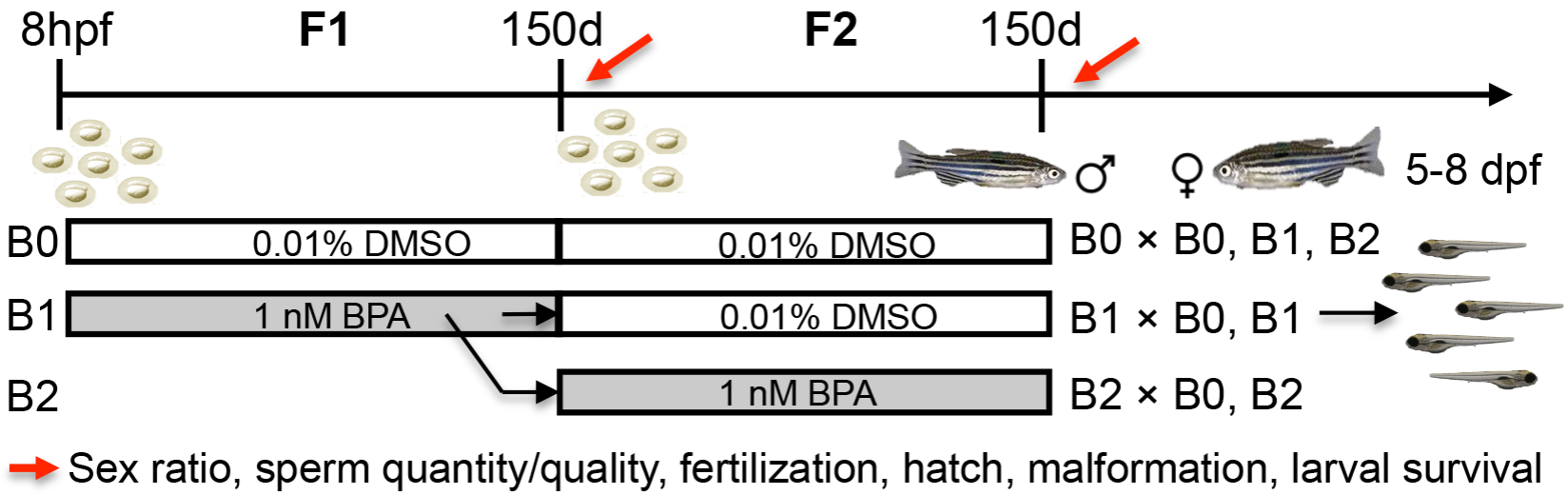
Primary Motoneuron (NBT-Tg; znp)



Secondary Motoneuron (zn-5)



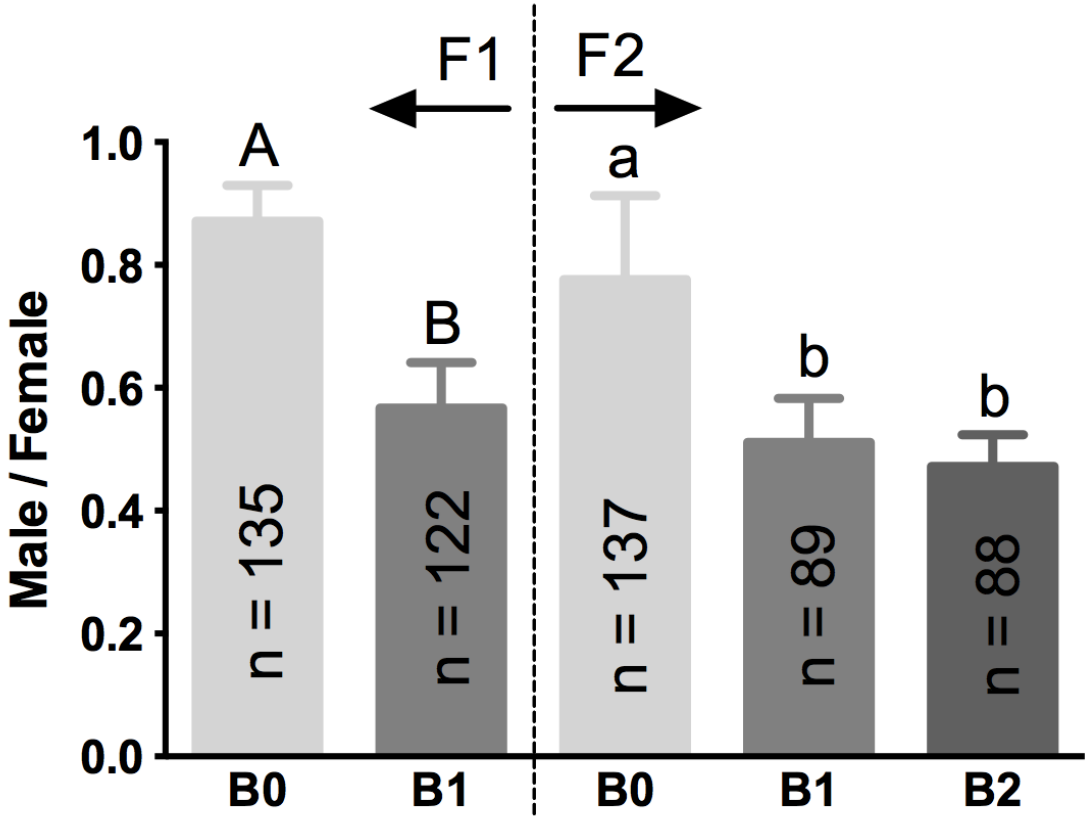
Two Generation Reproductive Toxicity



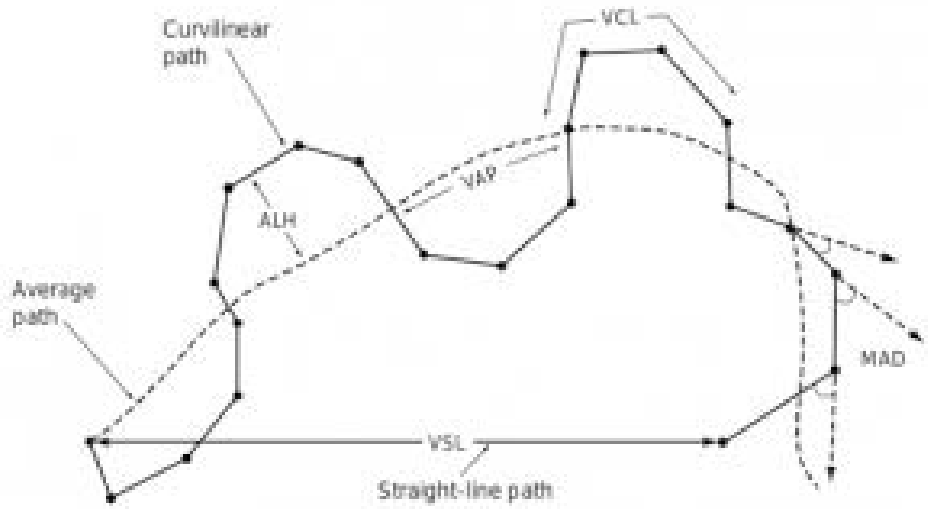
→ Sex ratio, sperm quantity/quality, fertilization, hatch, malformation, larval survival

Chen et al., Aquatic Tox, 2015, 169:204-214

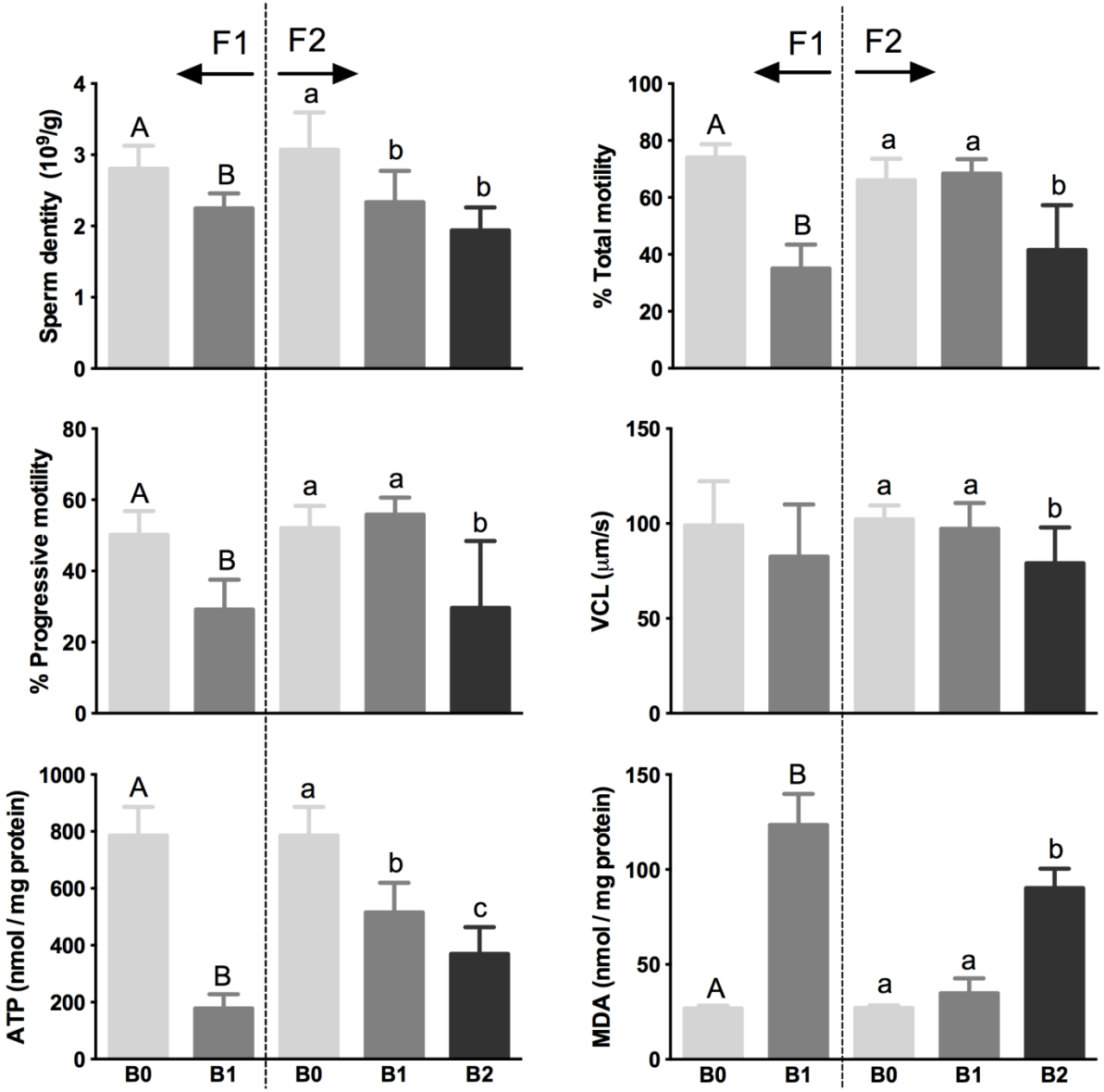
BPA Altered Sex Ratio



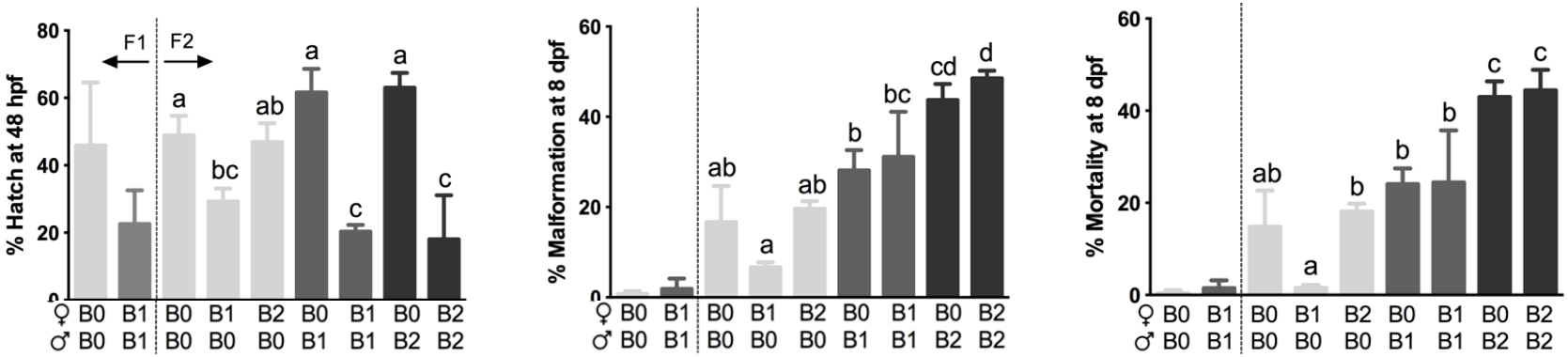
CASA: Computer-Assisted Sperm Analysis



BPA Affected Sperm Quality

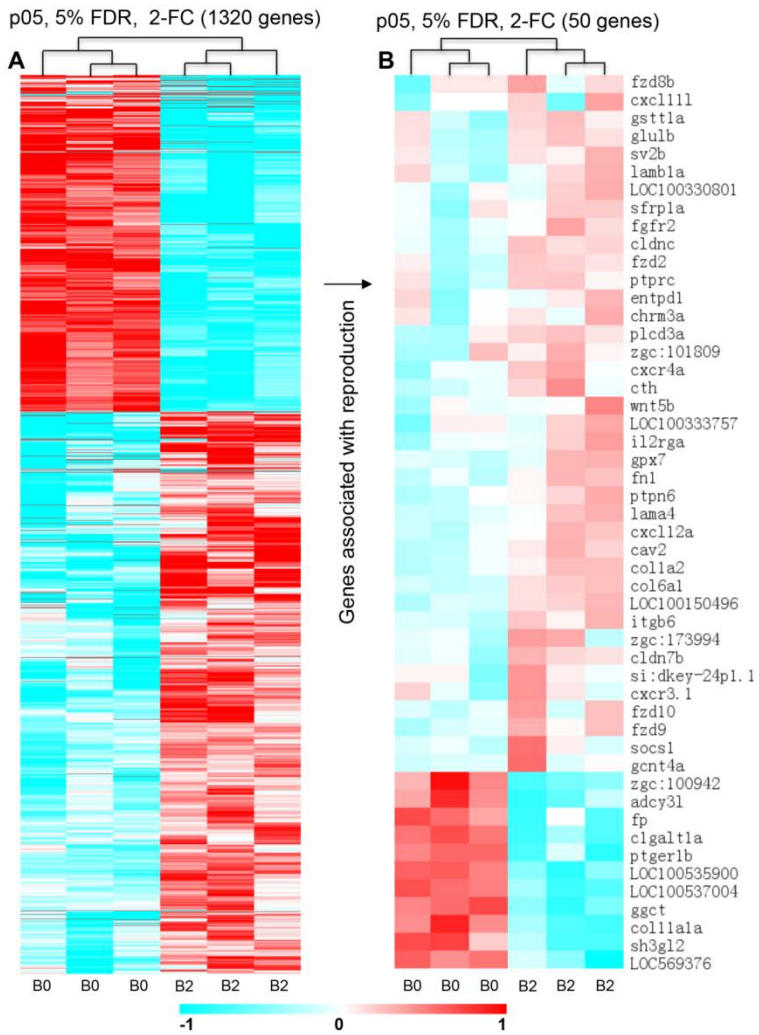


BPA Affected Hatch and Offspring Viability

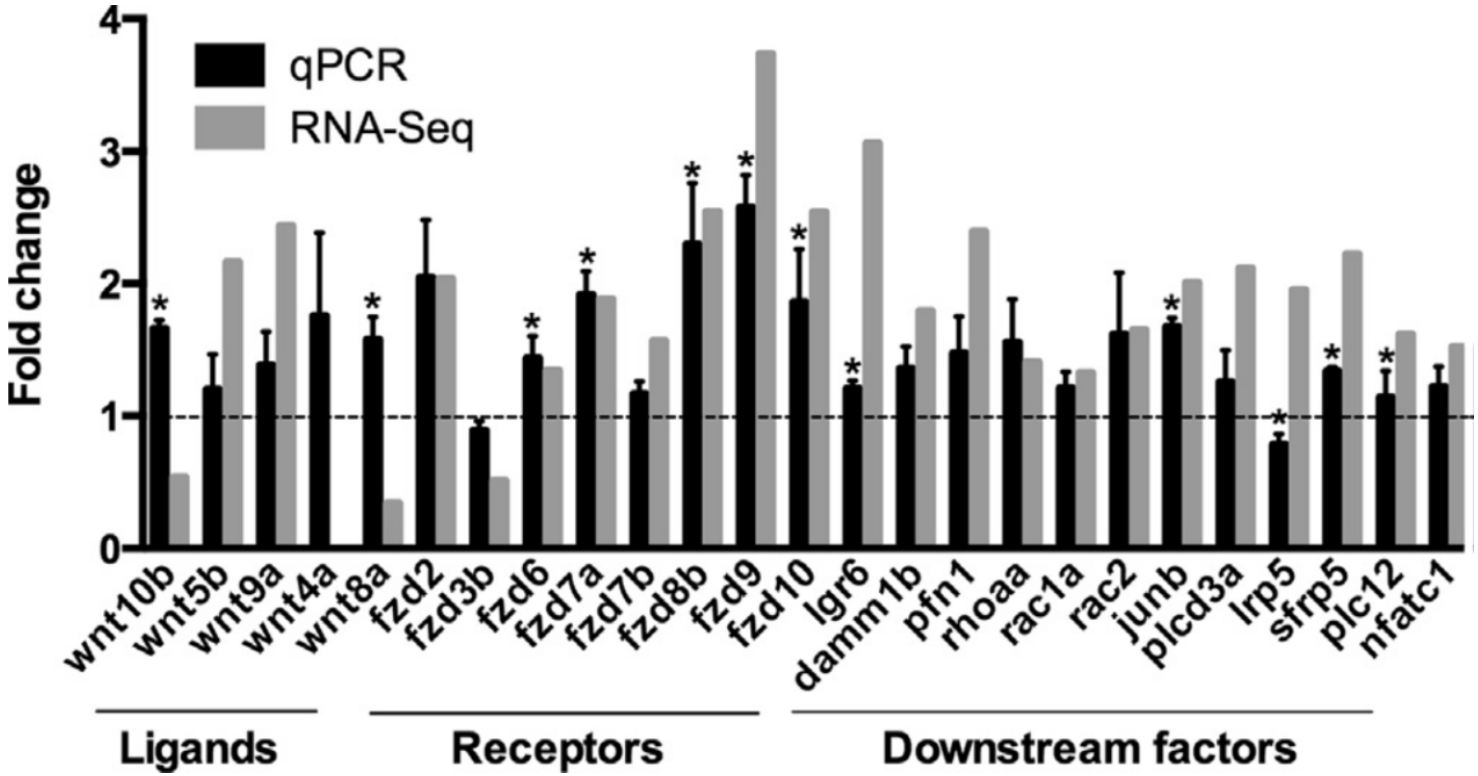


Maternal effect: Delayed hatch at 48 hpf
Paternal effect: Increased offspring mortality at 8 dpf

Transcription Analysis of Testis



Wnt Signaling Pathways

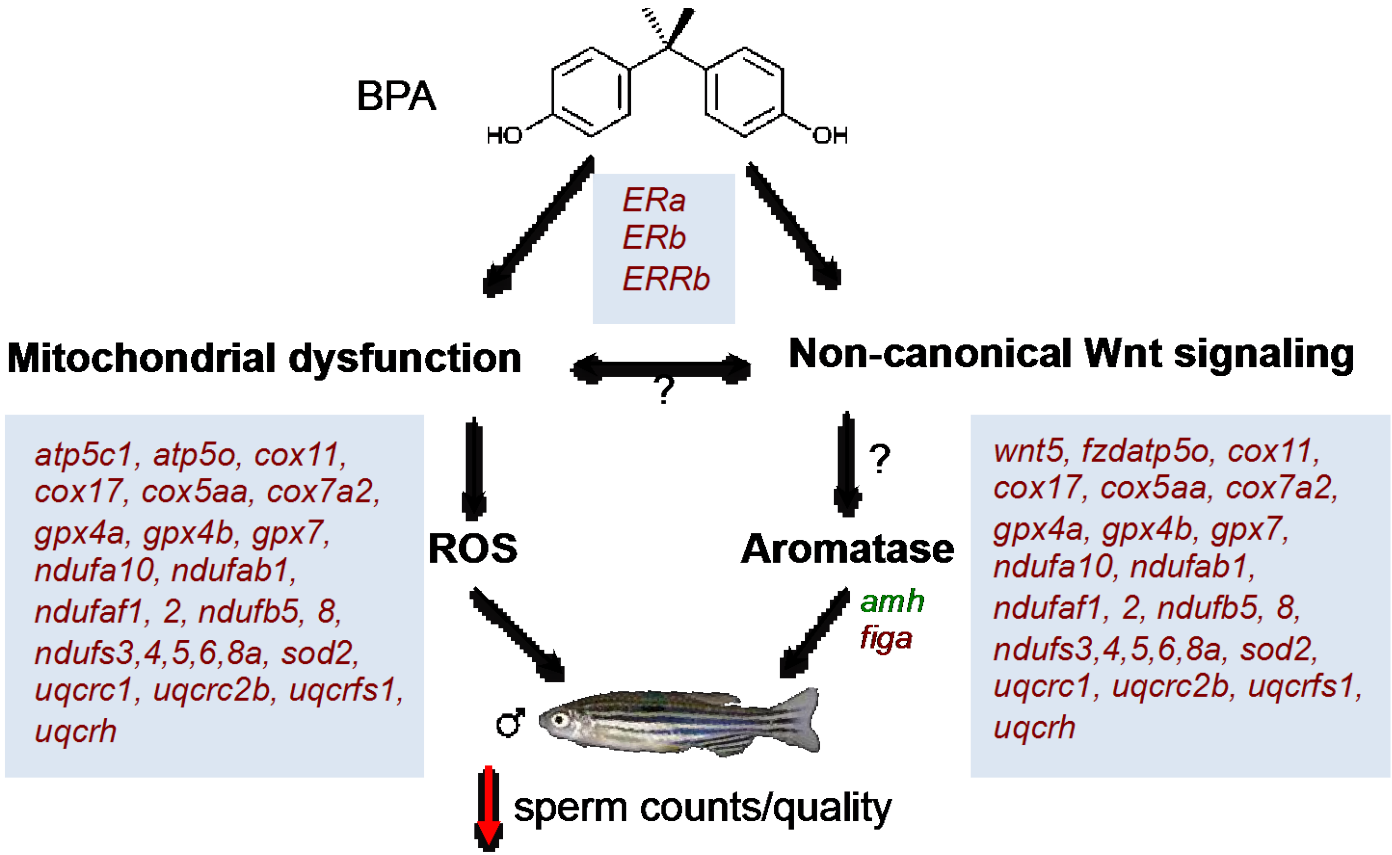


Mitochondrial Biogenesis

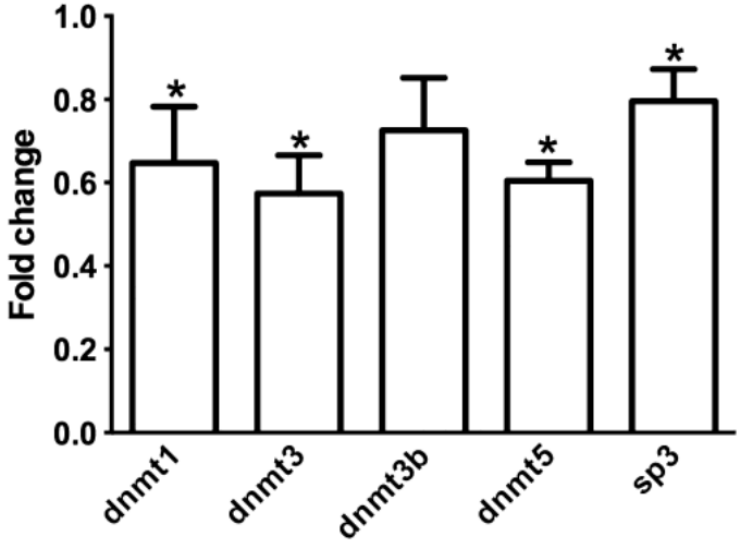
Table 2
Genes associated with mitochondrial biogenesis.

Symbol	Gene annotation	Log ratio	*P*-value
atp5c1	ATP synthase, mitochondrial F1 complex, gamma polypeptide 1	0.256	5.19E-07
atp5o	ATP synthase, mitochondrial F1 complex, O subunit	0.505	1.60E-06
cox11	COX11 cytochrome c oxidase copper chaperone	0.381	2.65E-03
cox17	COX17 cytochrome c oxidase copper chaperone	1.118	1.81E-04
cox5a	Cytochrome c oxidase subunit Va	0.596	3.47E-03
cox7a2	Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	0.776	8.37E-09
cyc1	Cytochrome c-1	0.204	3.28E-04
furin	Furin (paired basic amino acid cleaving enzyme)	-0.335	4.58E-03
gpx4	Glutathione peroxidase 4	0.440	2.22E-03
gpx7	Glutathione peroxidase 7	2.240	1.22E-02
map2k4	Mitogen-activated protein kinase kinase 4	-0.936	1.54E-05
mt-co2	Cytochrome c oxidase subunit II	1.448	2.03E-02
mt-co3	Cytochrome c oxidase III	1.229	6.78E-03
mt-cyb	Cytochrome b	1.584	8.84E-03
mt-nd1	NADH dehydrogenase, subunit 1 (complex I)	1.447	4.86E-02
ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	0.404	5.49E-16
ndufab1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex 1	0.877	4.13E-03
ndufaf1	NADH dehydrogenase (ubiquinone) complex I, assembly factor 1	0.337	1.74E-02
ndufaf2	NADH dehydrogenase (ubiquinone) complex I, assembly factor 2	0.754	1.34E-04
ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5	0.825	2.83E-03
ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	0.433	4.48E-02
ndufs3	NADH dehydrogenase (ubiquinone) Fe–S protein 3	0.466	9.23E-06
ndufs4	NADH dehydrogenase (ubiquinone) Fe–S protein 4	0.727	9.38E-05
ndufs5	NADH dehydrogenase (ubiquinone) Fe–S protein 5	0.426	3.11E-03
ndufs6	NADH dehydrogenase (ubiquinone) Fe–S protein 6	1.066	2.33E-03
ndufs8	NADH dehydrogenase (ubiquinone) Fe–S protein 8	0.452	6.51E-06
park7	Parkinson protein 7	0.624	2.58E-12
prdx3	Peroxiredoxin 3	0.702	2.03E-31
prdx5	Peroxiredoxin 5	1.045	1.40E-06
sdhc	Succinate dehydrogenase complex, subunit C	0.635	9.82E-08
sod2	Superoxide dismutase 2, mitochondrial	0.778	5.71E-05
surf1	Surfeit 1	0.621	4.51E-04
uqcrc1	Ubiquinol-cytochrome c reductase core protein I	0.451	4.15E-24
uqcrc2	Ubiquinol-cytochrome c reductase core protein II	0.235	3.52E-02
uqcrfs1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	0.563	9.33E-04
uqcrh	Ubiquinol-cytochrome c reductase hinge protein	0.409	7.68E-08

Mechanisms for Reduced Sperm Quality



Epigenetic Changes for Male-mediated Offspring Mortality



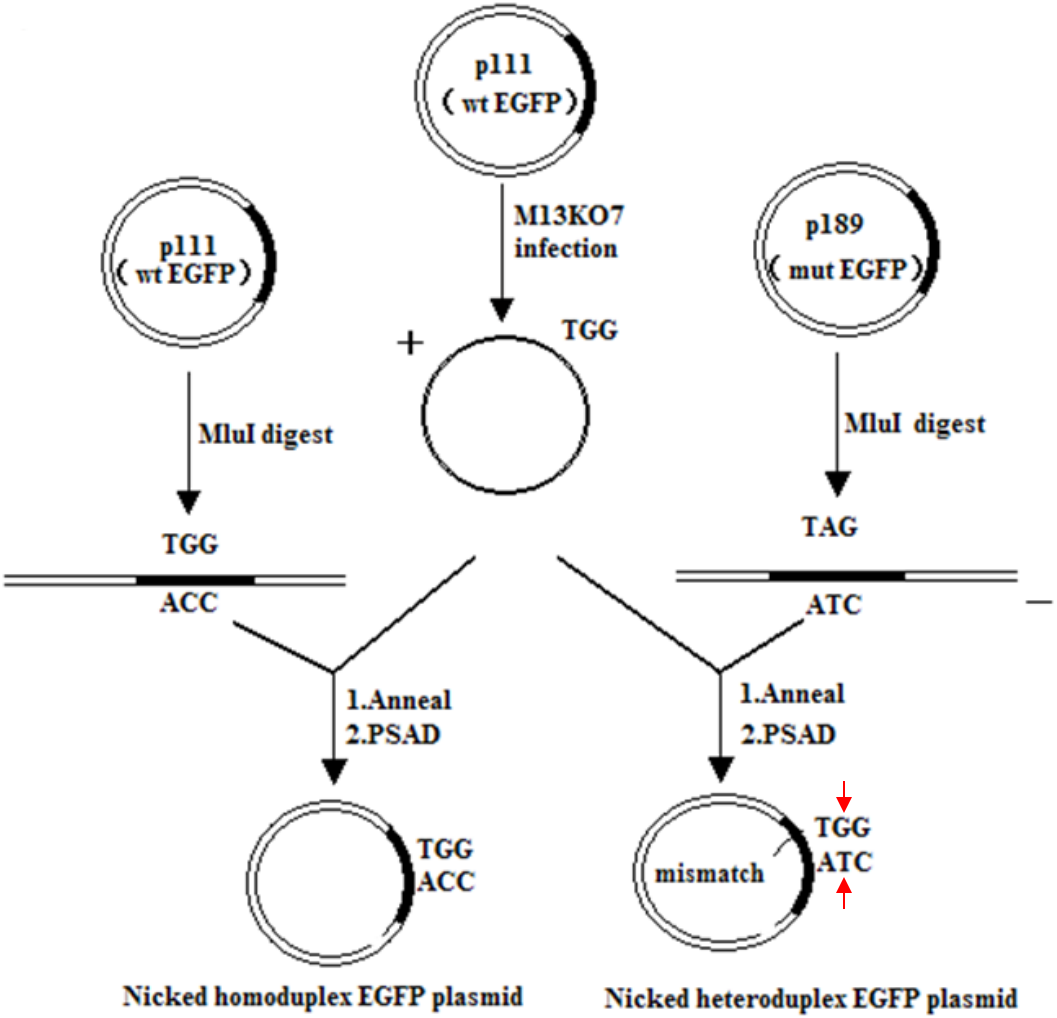
DNA methytransferases (Dnmts)

Paternal mediated post-implantation loss

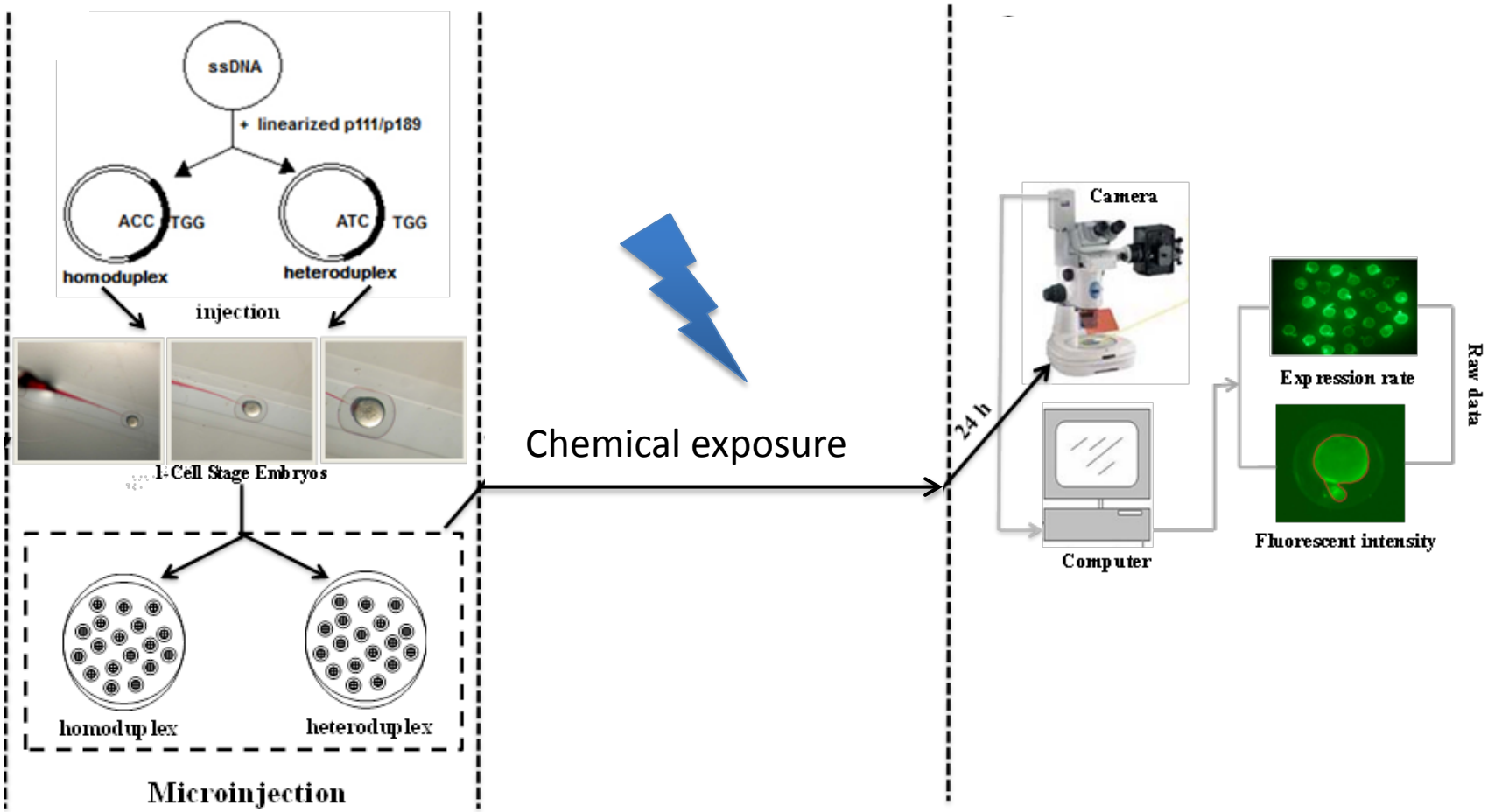
- Rat
- Neonatal BPA exposure
- BPA-exposed males
- Resorbed embryos vs. viable embryos
- Down regulated Dnmt1, Dnmt3a, Dnmt3b
- Down regulated Sp1 and Sp3

Doshi et al., 2012, J Biochem Mol Toxicol, 26: 337-343

In vivo DNA Mismatch Repair

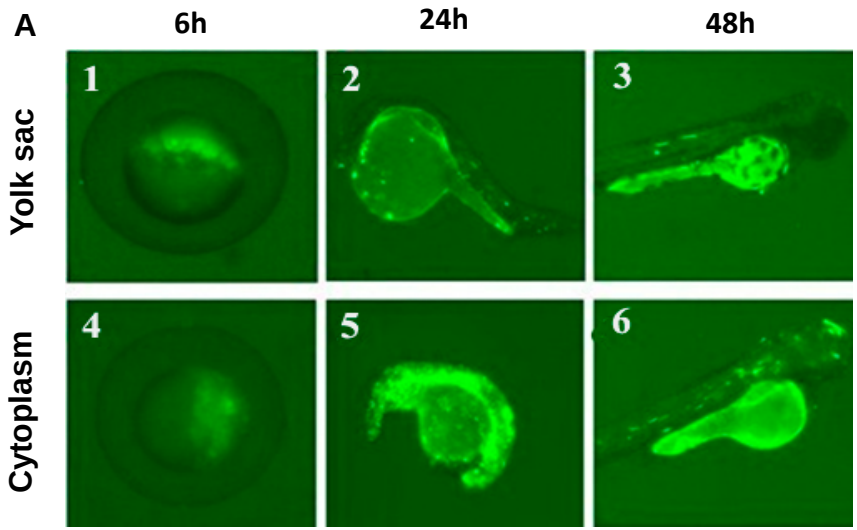


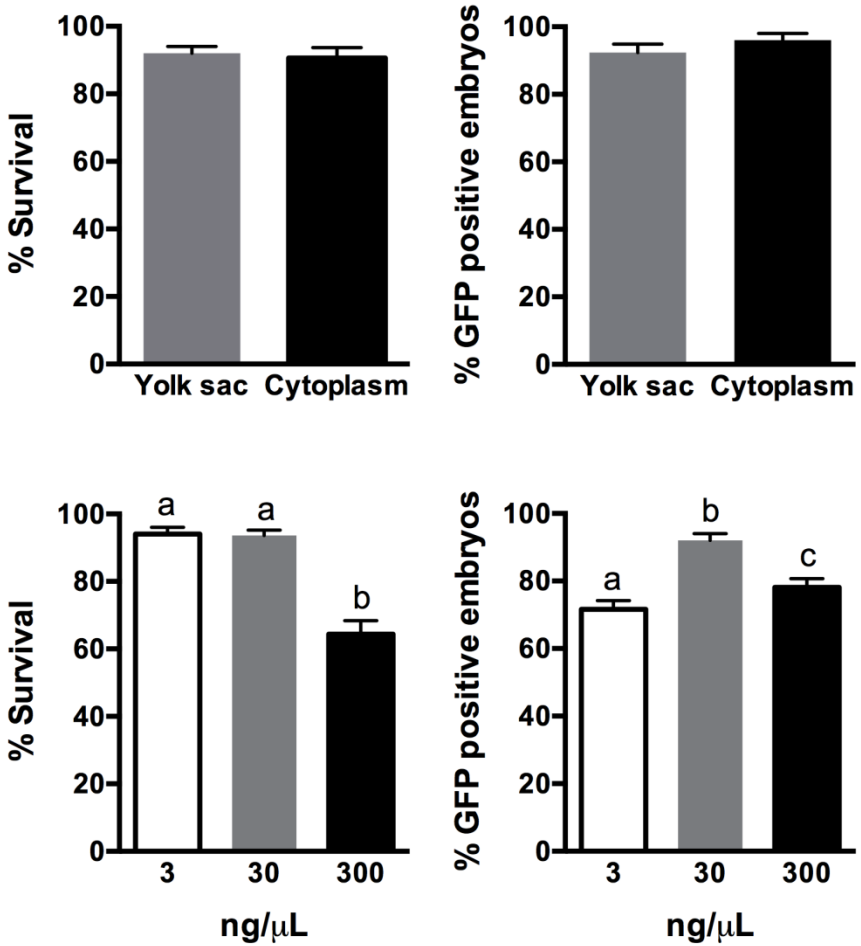
In vivo DNA MMR in Zebrafish



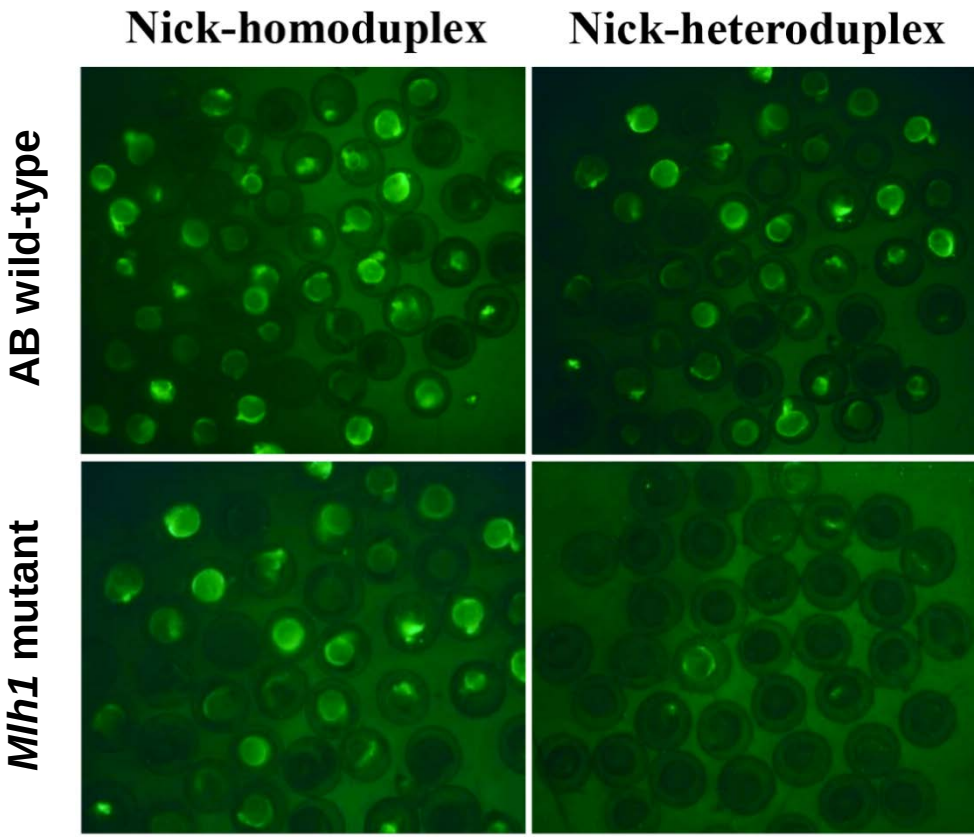
Chen et al., J Hazardous Mat, 2016, 302:296-303

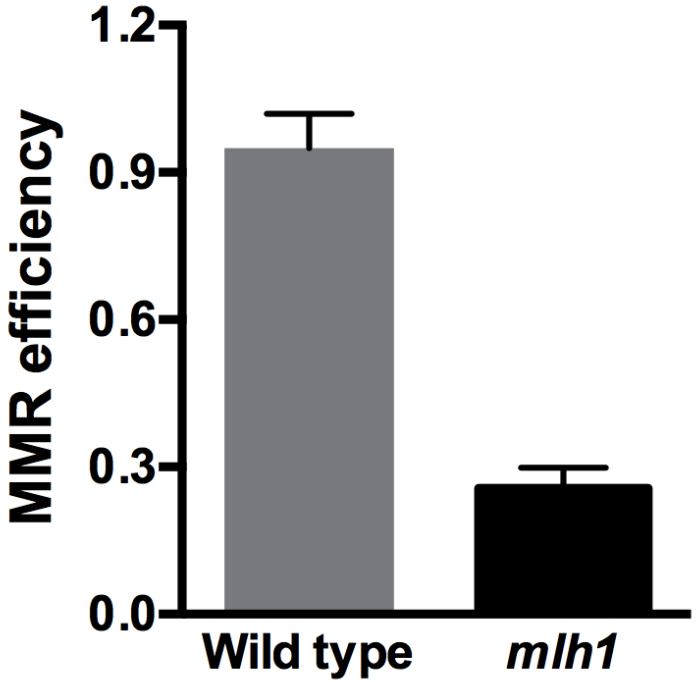
Injection Site and Dose





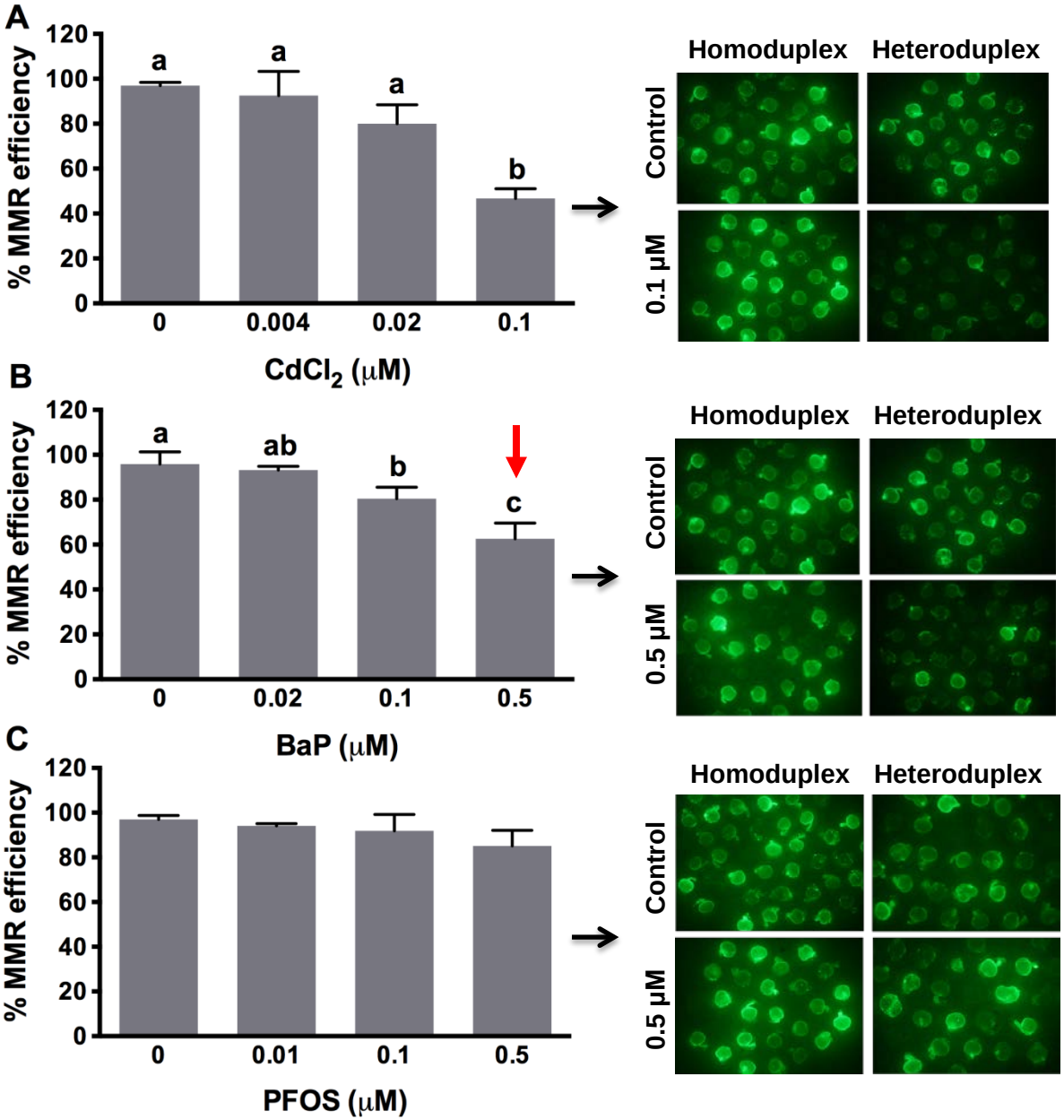
Validation with *Mlh1* mutant



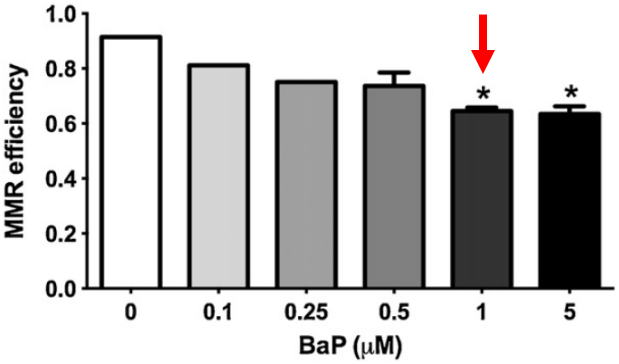


Mlh1+/- was a gift from Prof. Edwin Cuppen at the University of Utrecht (Netherlands)

Application and Sensitivity



Breast cancer cell line ZR75-1

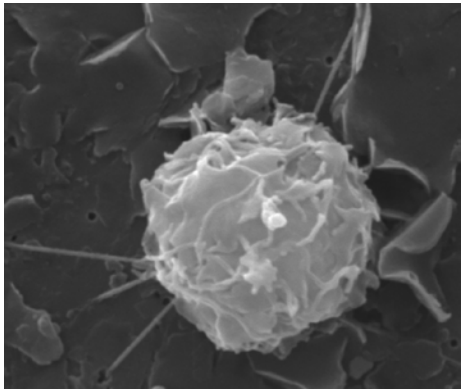


Chen et al., Toxicology 2013, 304:167-172

Zebrafish Model

- Advantages
  - Effects are systemic, metabolic or behavioral
  - All the tools available for developing Tg fish for mode of action risk assessment
  - Can be used in high throughput setting
  - Complementary to HTS using in vitro cell assays
  - Very relevant for ecotoxicological risk assessment
- Challenge
  - How to extrapolate these data for human health risk assessment (a fish is a fish)

Mouse Mammary Stem Cells



C57BL6/J
(WT, GFP, DsRed)

FVB (WT, GFP)

Balb/C (WT, GFP)

Target cells: stem/progenitors

- Long-lived (accumulate DNA damage)
- Multi- or unipotent (tumor heterogeneity)
- Self-renewal (tumor expansion)
- Developmental sensitivity (stem cell expansion in earlier life stage)
- Early exposure and later onset of disease
  - Atomic bomb, most susceptible age group 8-13, breast cancer onset after 50

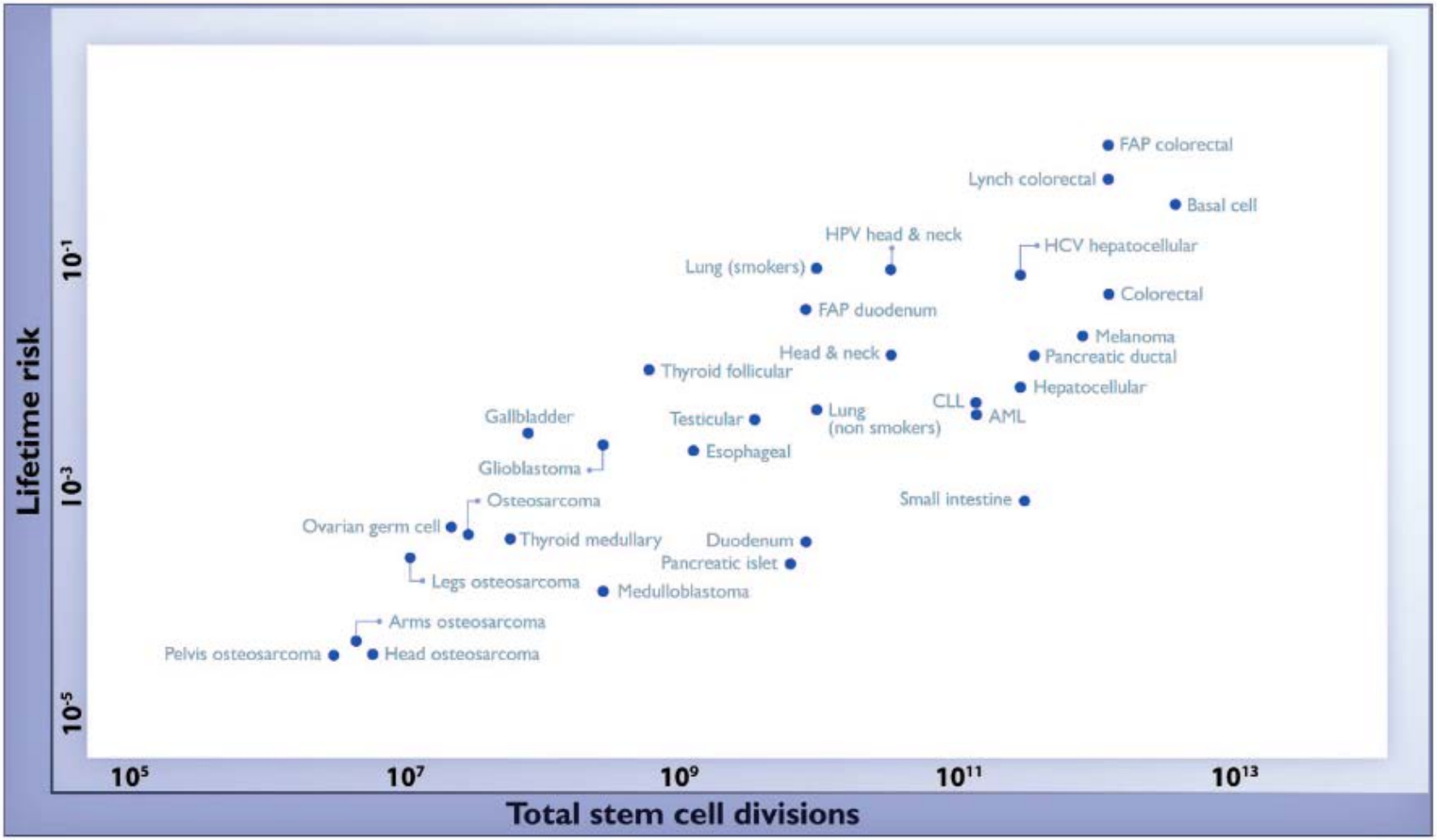
used by eukaryotes, which lack tmRNA, to recognize and rescue stalled ribosomes and their incomplete translation products have been unclear. The RQC—and Rqc2p's CAT tail tagging mechanism in particular—bear both similarities and contrasts to the tmRNA *trans*-translation system. The evolutionary convergence upon distinct mechanisms for extending incomplete nascent chains at the C terminus argues for their importance in maintaining proteostasis. One advantage of tagging stalled chains is that it may distinguish them from normal translation products and facilitate their removal from the protein pool.

CANCER ETIOLOGY

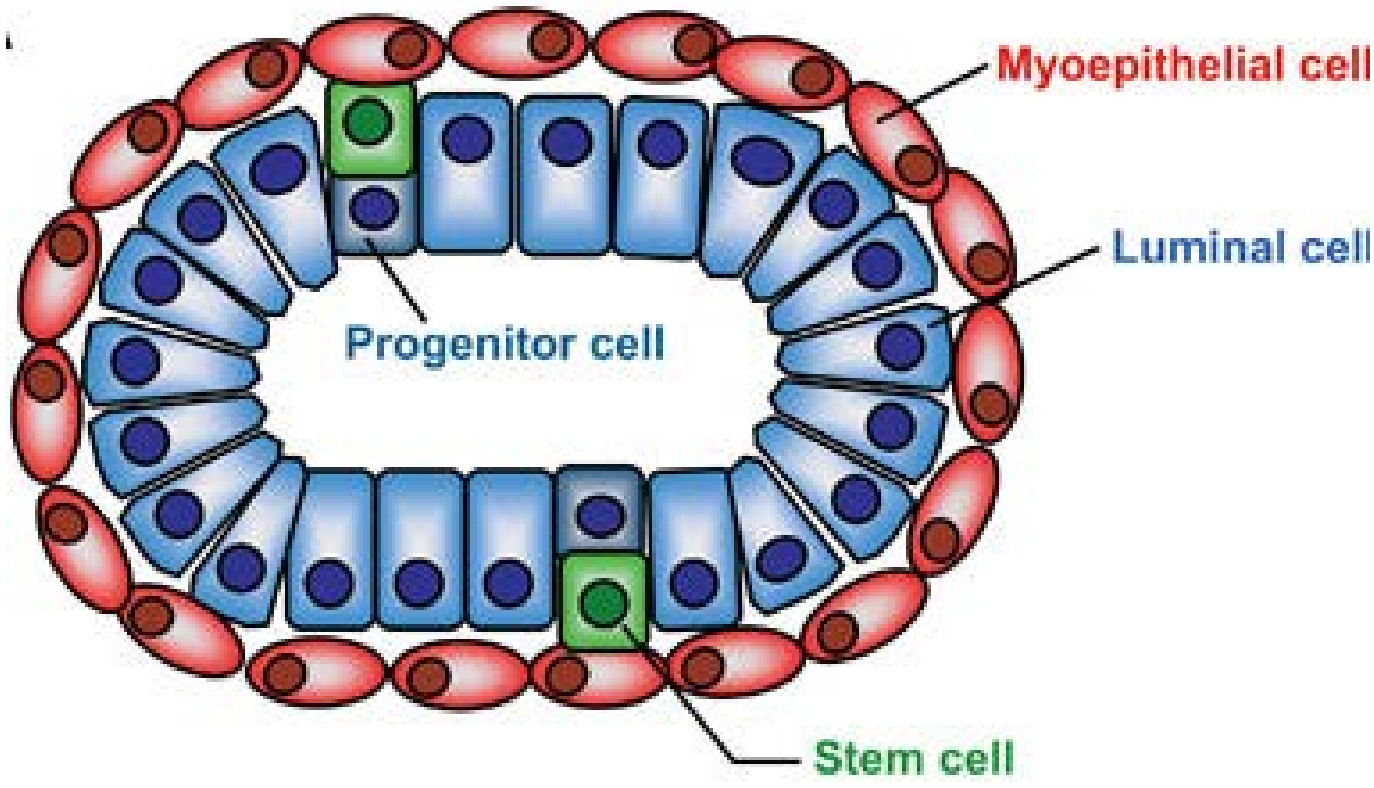
Variation in cancer risk among tissues can be explained by the number of stem cell divisions

Cristian Tomasetti[1]* and Bert Vogelstein[2]*

/.sciencemag.org on Januar

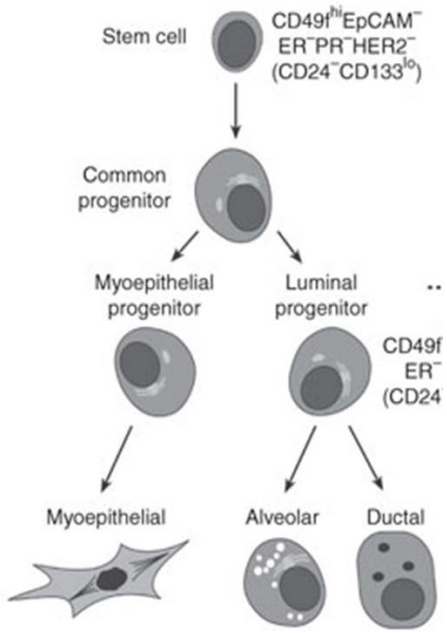


Mammary Duct

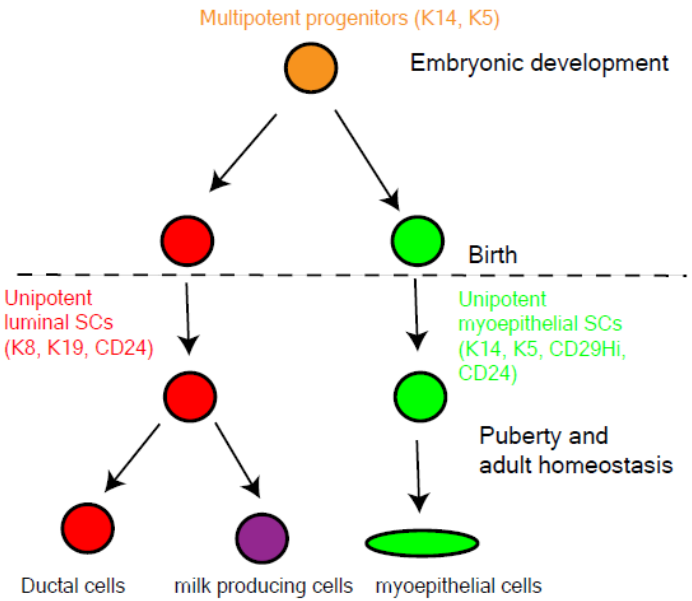


Tiede and Kang, 2011, Cell Res

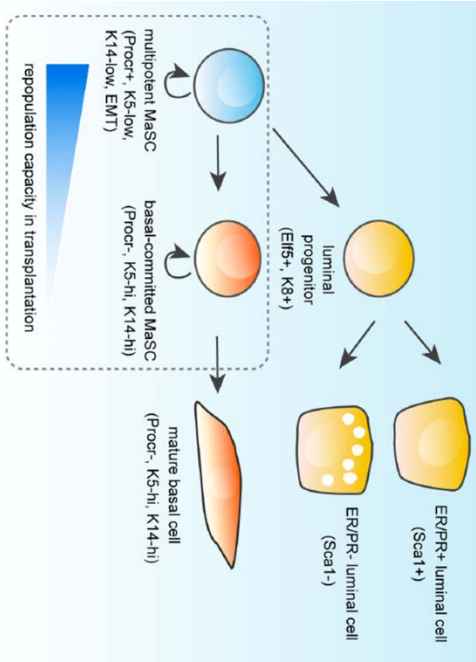
Mammary Stem Cell Hierarchy



Lim et al., 2009, Nat Med 15:907-13

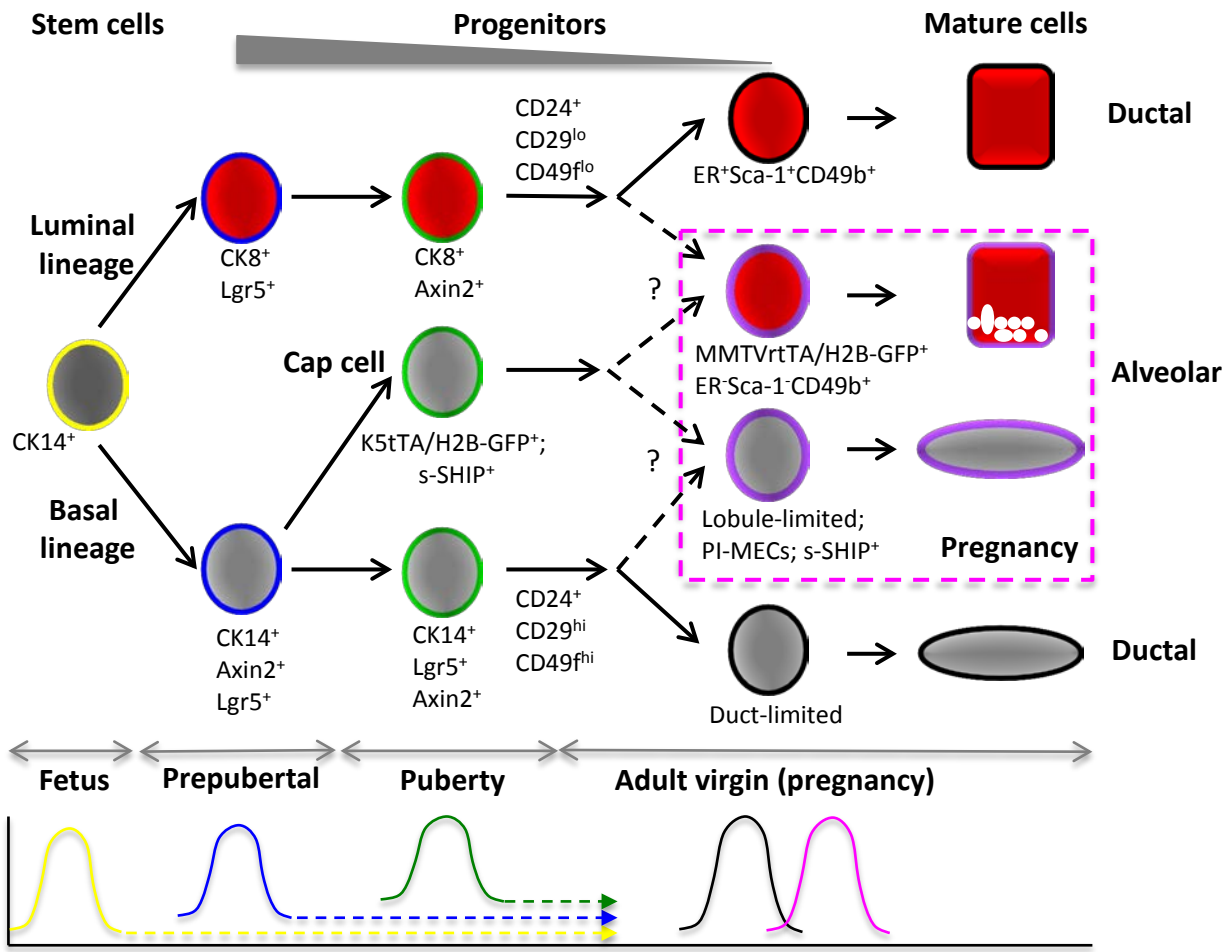


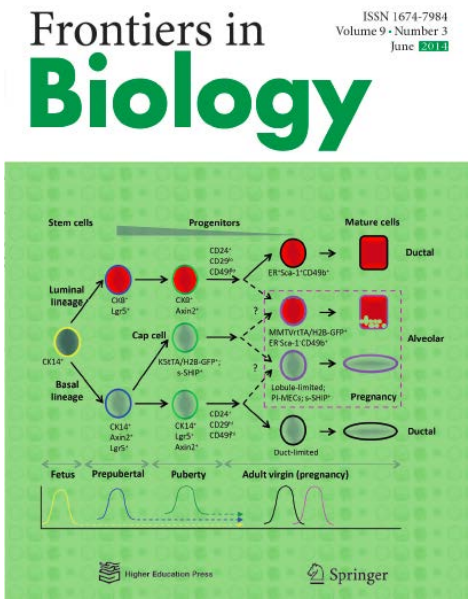
Van Keymeulen et al., 2011, Nature



Wang et al., 2014, Nature

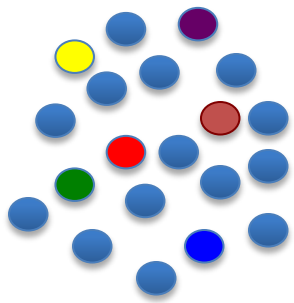
Our hypothetical cell hierarchy model

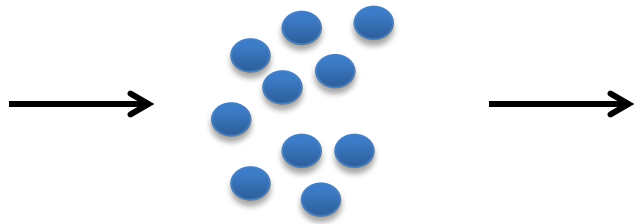


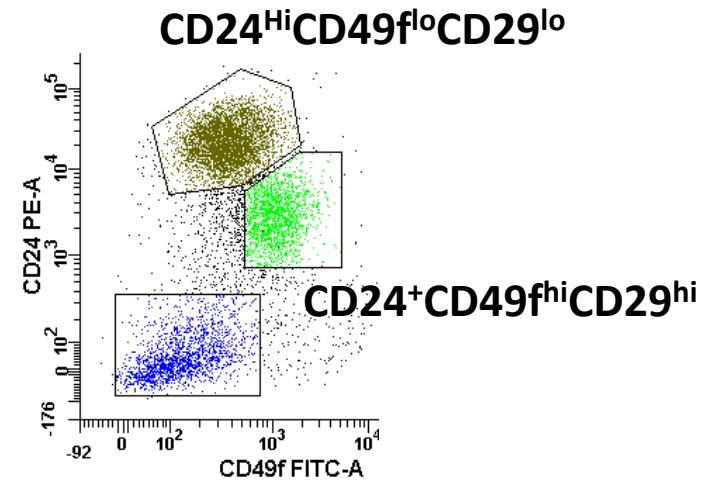


Dong and Sun, 2014, Frontiers in Biology

Stem Cell Enrichment and Identification





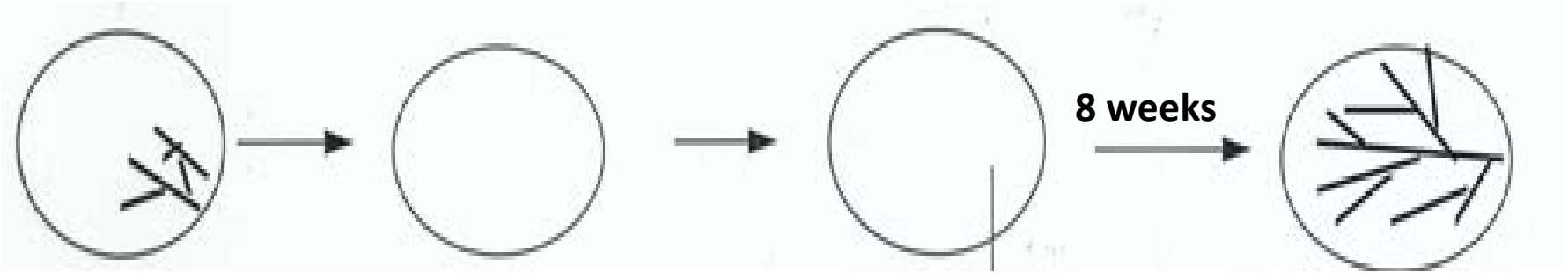


Lin-(CD31-CD45-TER119-)

CD24/EpCAM/CD49f/CD29

- Identification: in vivo fat pad transplant (IVT)
- Quantification: limiting dilution IVT
- Self-renewal: series IVT
- Differentiation: lobulo-alveolar genesis and milk production
- Luminal MaSC: in vitro 2D colony formation (2D CFC)

In vivo Cleared Fat Pad Transplant (Basal MaSC)

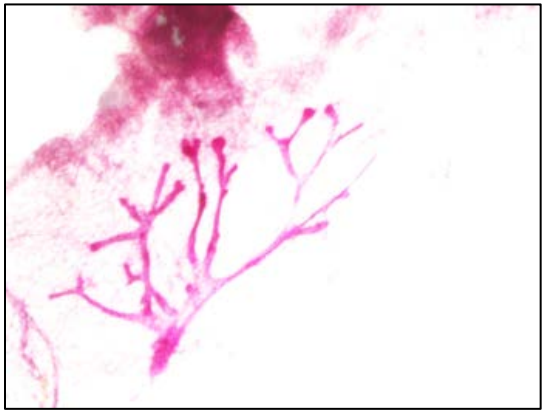


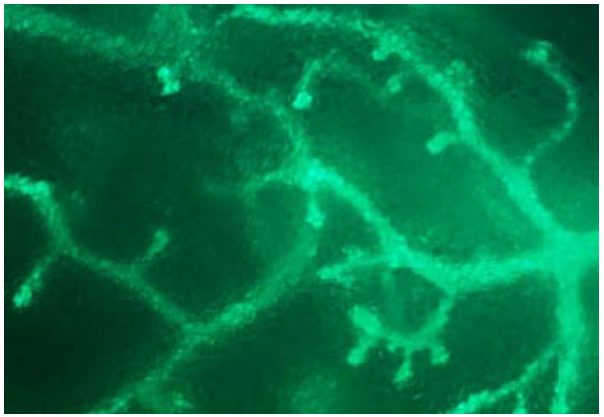
3 week old

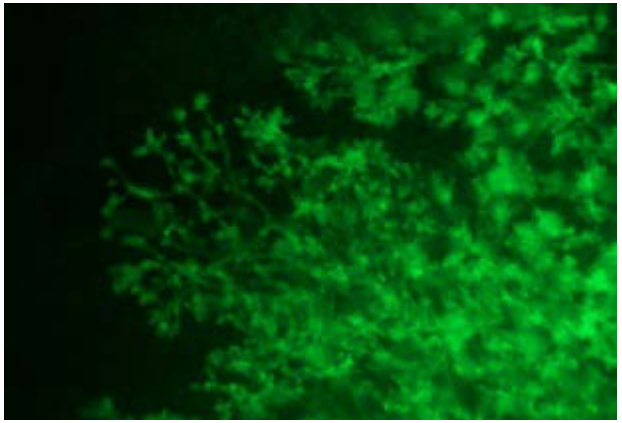
Cleared Fat Pad (CFP)

Injected with MaSC

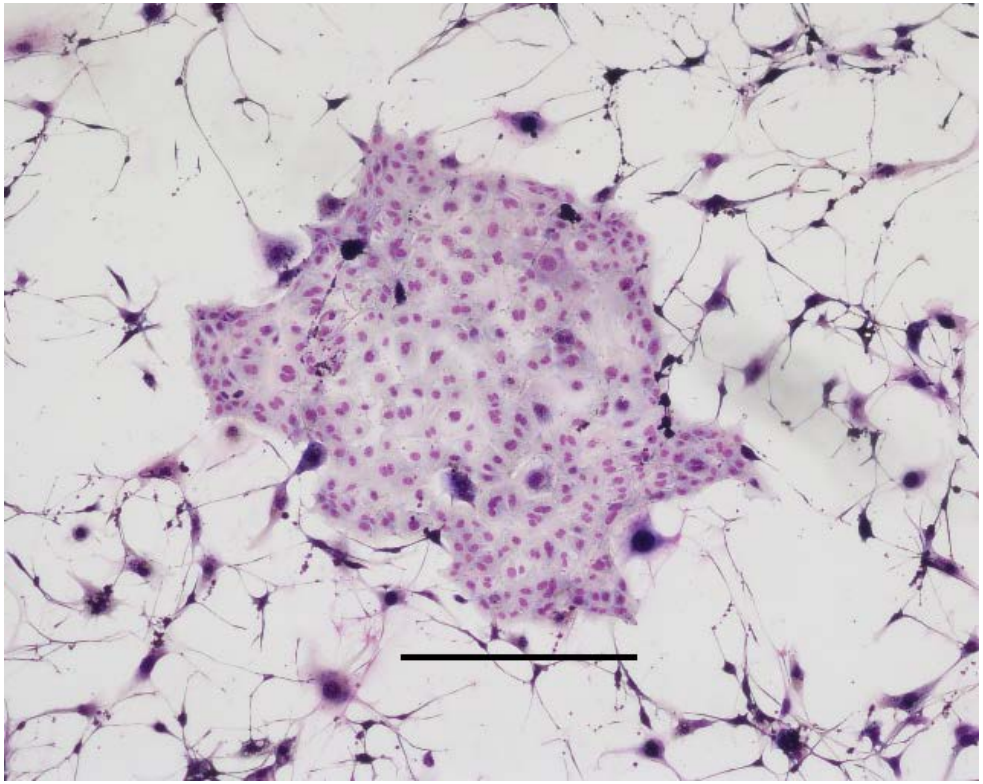
Regenerated gland



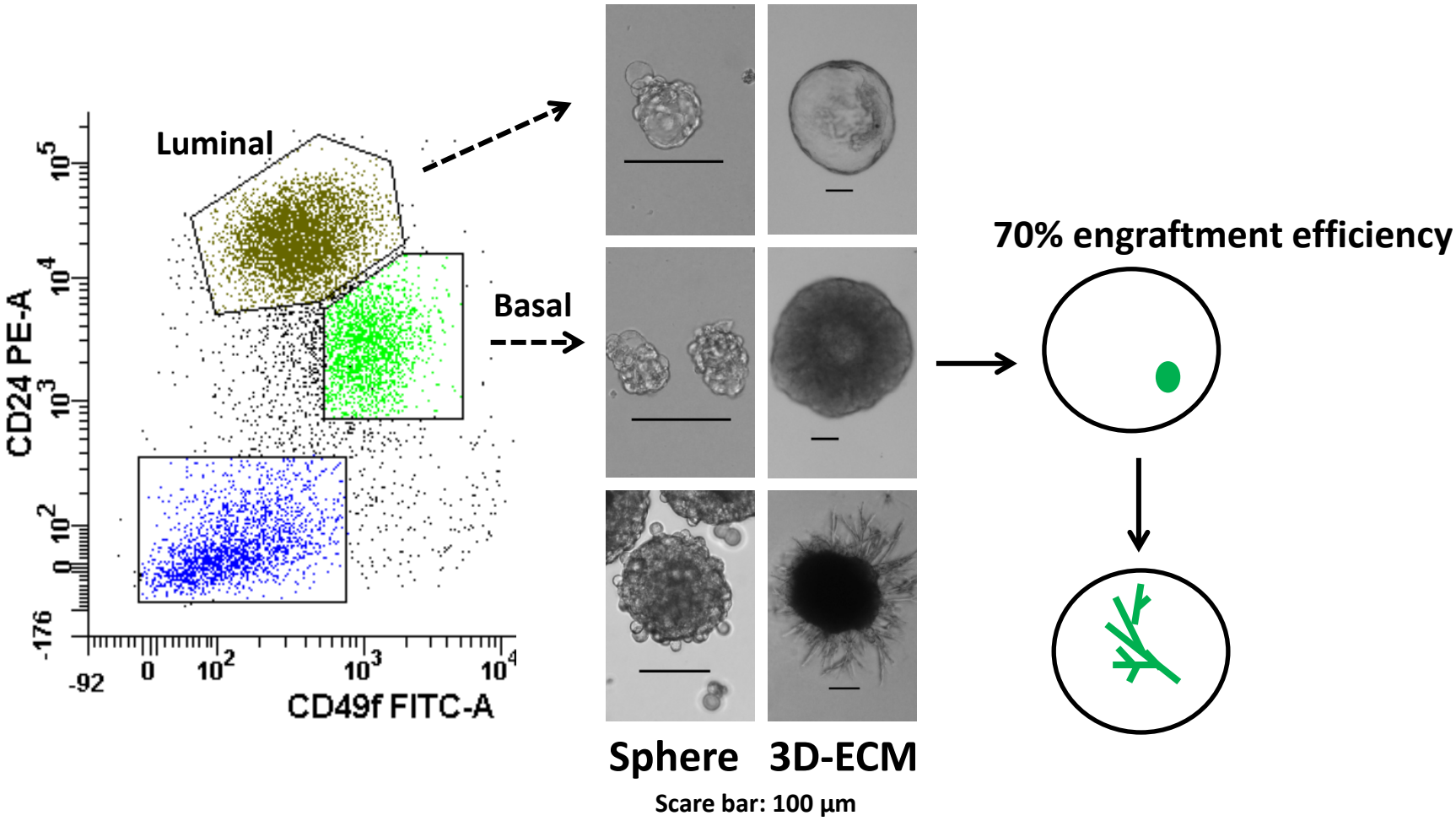




In vitro Colony Forming Cell Assay (Luminal MaSC/progenitor)



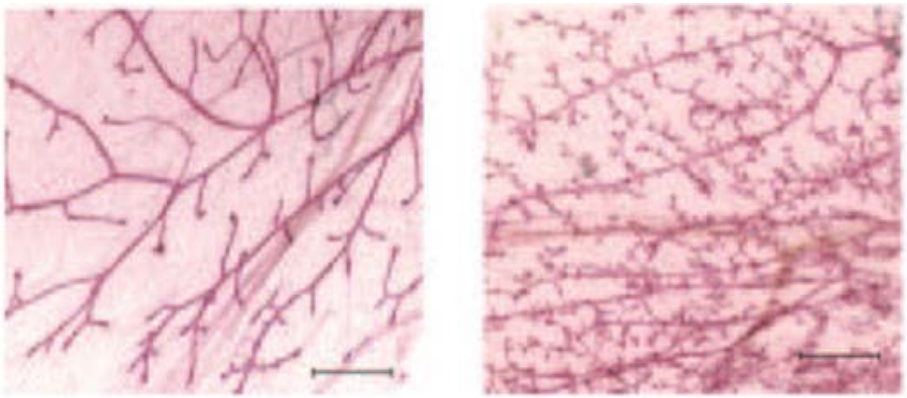
Sphere Formation and Differentiation (Basal MaSC and Luminal Progenitor)



Dong et al., 2013, Stem Cell Res

Perinatal BPA Exposure and Mammary Gland Morphogenesis/Tumor

CD1 mouse

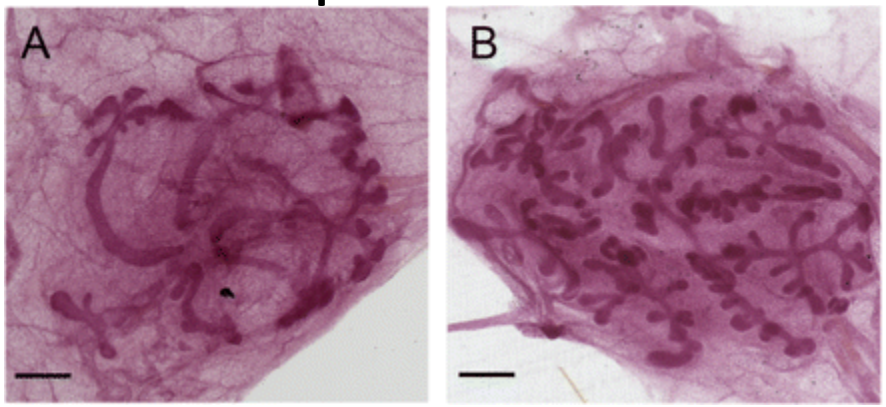


Munoz-de-Toro et al. 2005, Endocrinology 146: 4138-47

Accelerated puberty onset
Prolonged estrous cycle

Terminal ducts/TEB
Lateral branches
Epithelial cell number

Rhesus macaque



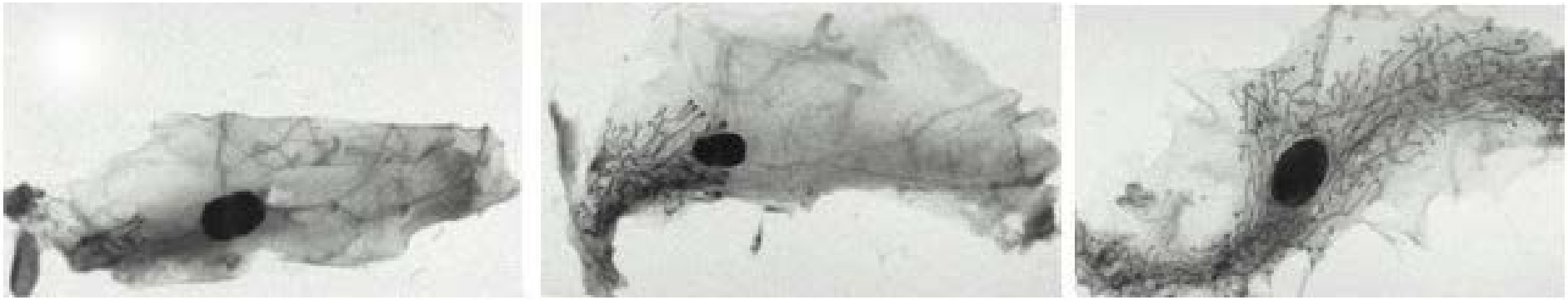
Tharp et al., 2012, PNAS 109:8190-95

Endogenous estrogen
Progesterone response

Preneoplastic ductal hyperplasias
Carcinoma in situ
DMBA induced tumorigenesis

Pubertal BPA Exposure

Newborn | **3-week old** | **6-week old**

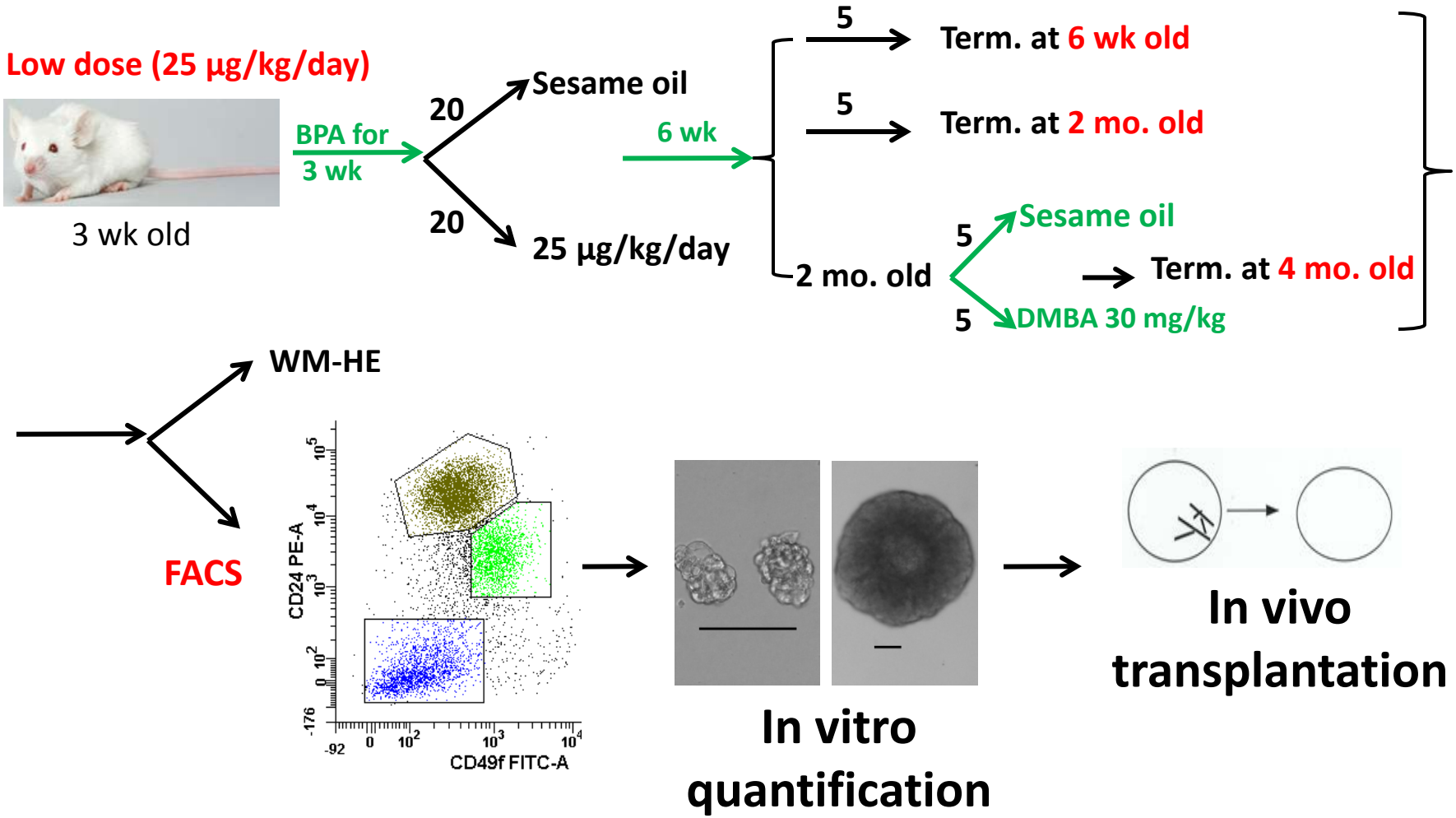


A susceptible window for MaSC

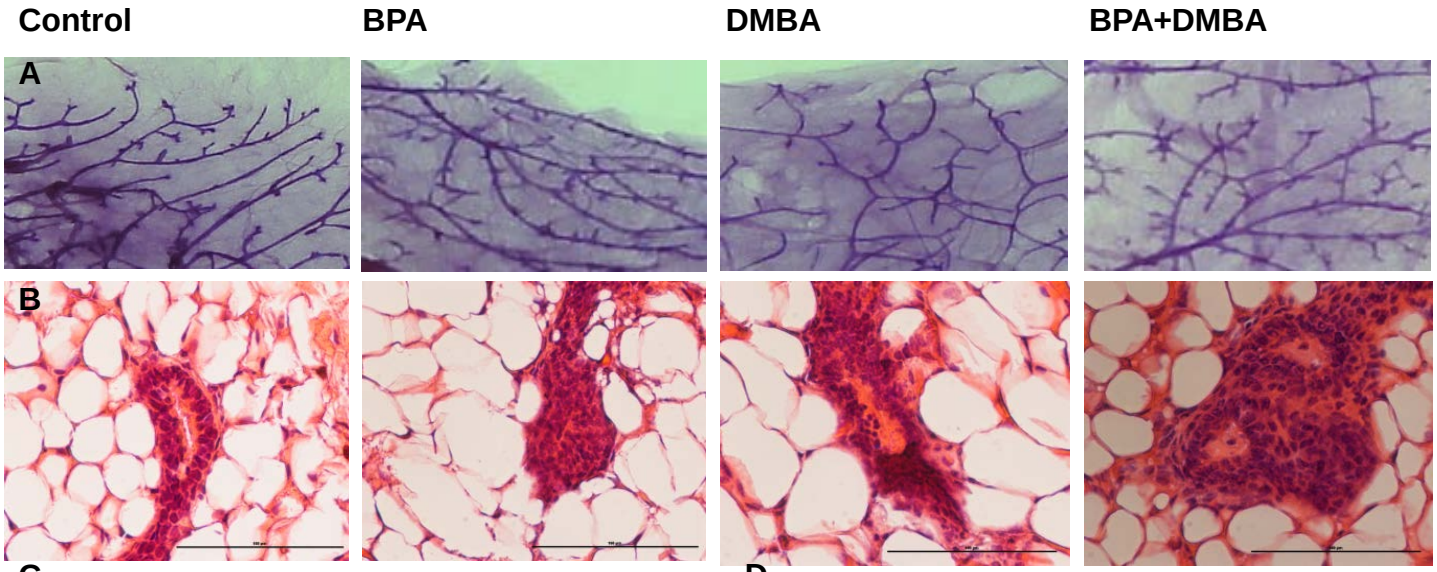
- ❖ **Puberty: Week 4-Week 6**
- ❖ **Extensive ductal elongation**
- ❖ **MaSC proliferative state**

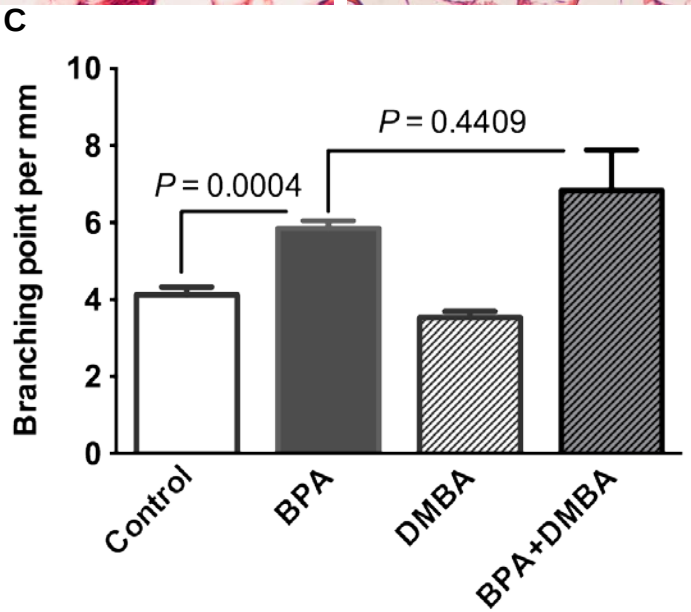
http://mammary.nih.gov/index.html

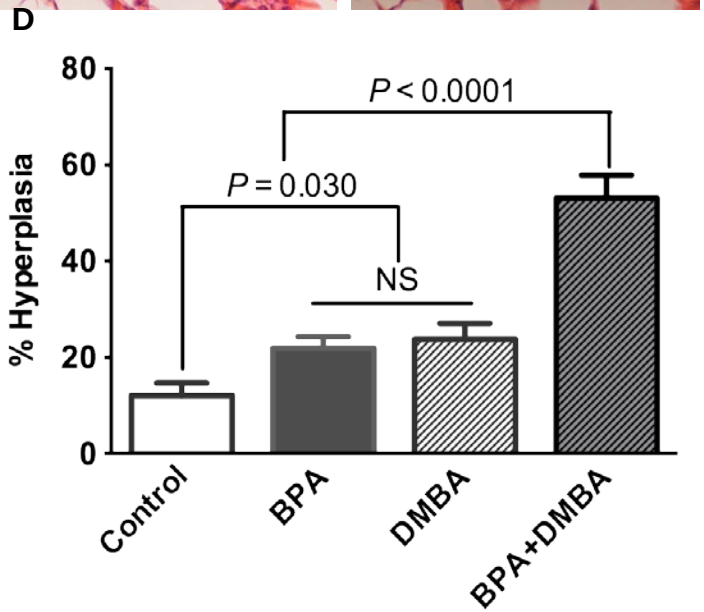
Experimental Scheme



BPA/DMBA Increased Hyperplasia in 4-month Old Glands



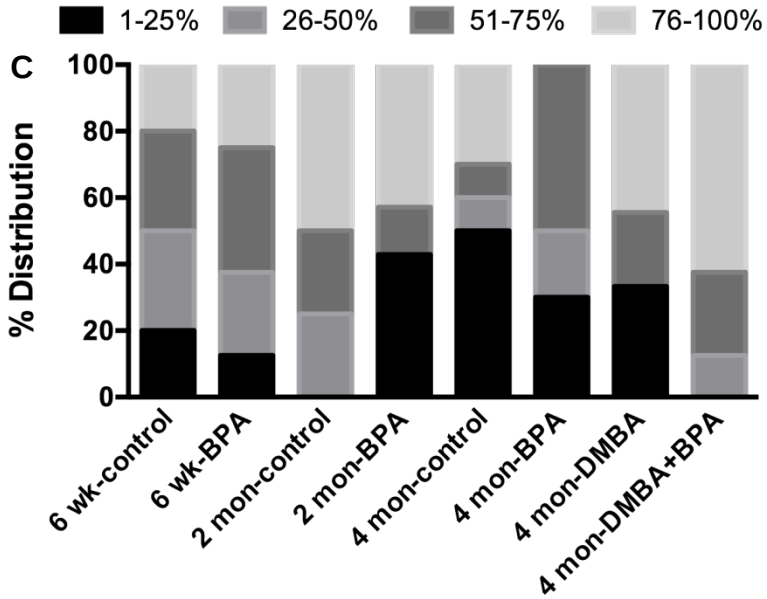


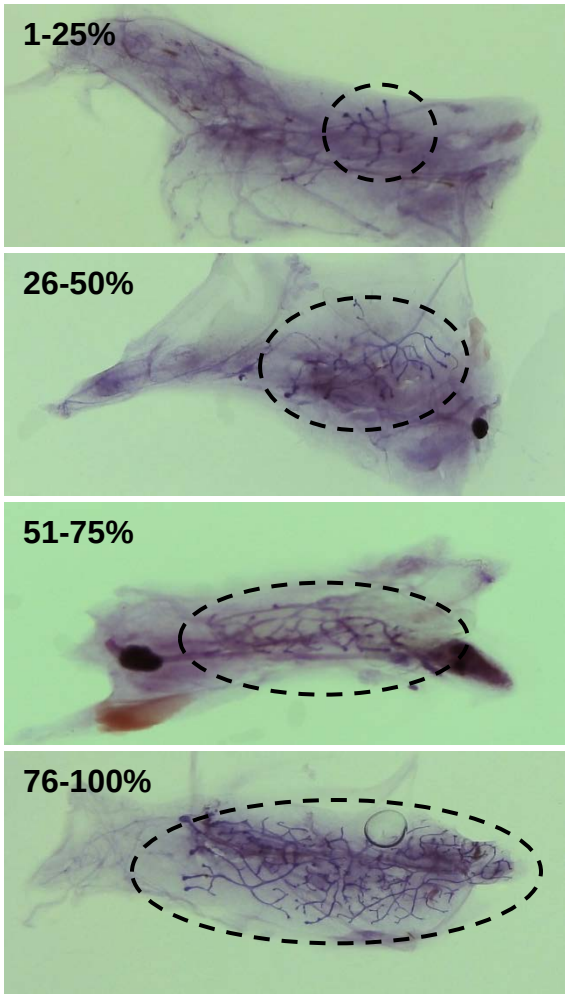


BPA did NOT Affect Basal MaSCs Repopulation Capability

In vivo cleared fat pad transplant (IVT)

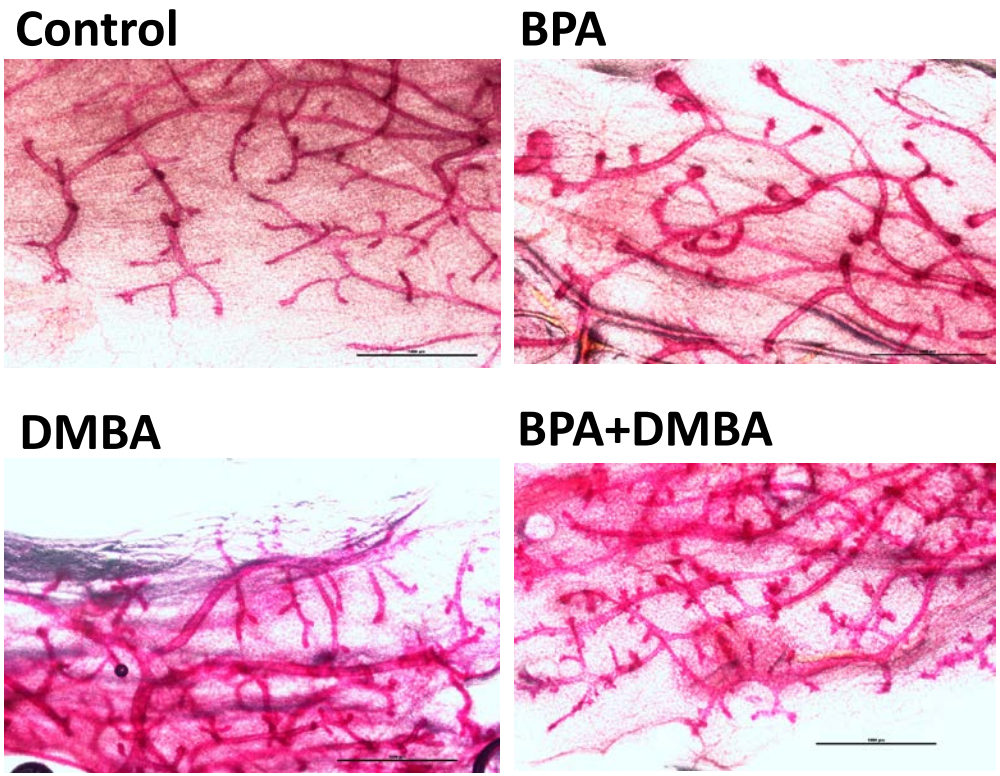
	Positive/total CFP	
Termination time point	**Control**	**BPA**
6-week	10/10	8/10
2-month	4/11	7/11
4-month without DMBA	10/10	10/10
4-month with DMBA	9/10	8/10

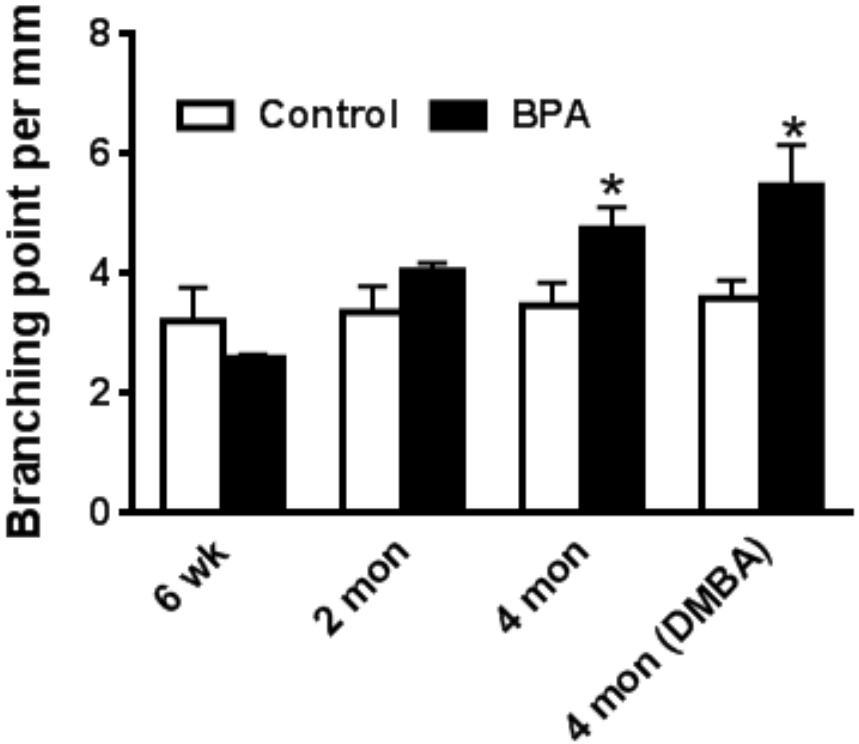




Wang et al., 2014, Cancer Prev Res

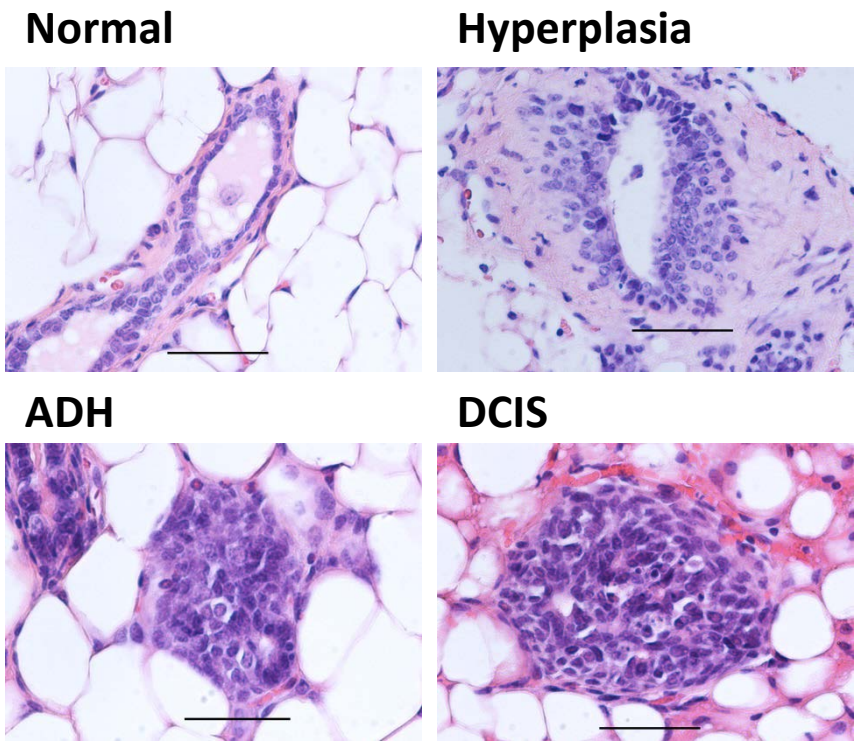
Pubertal BPA Exposure Increased Lateral Branching in Regenerated Glands

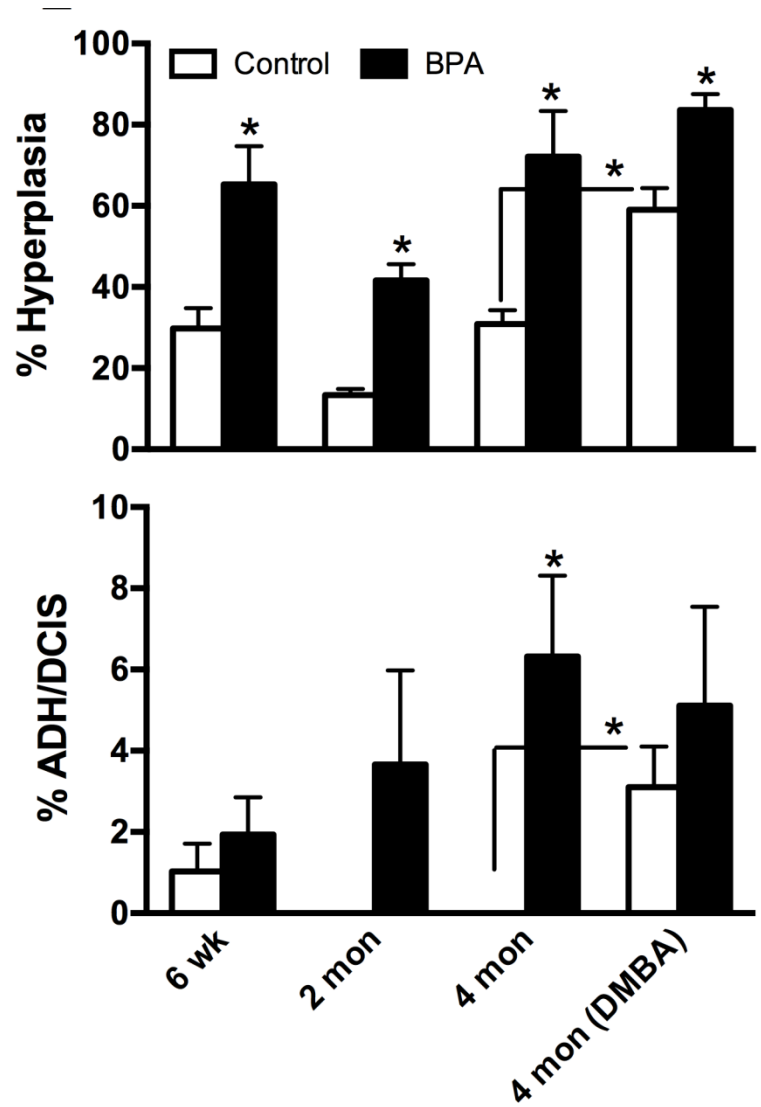




Wang et al., 2014, Cancer Prev Res

Increased Hyperplasia in Regenerated Glands from BPA Exposed MaSCs





Wang et al., 2014, Cancer Prev Res

RNAseq on MaSC-enriched Spheres

Low dose (25 µg/kg/day)

3 wk old

BPA for 3 wk → 20 → Sesame oil; 20 → 25 µg/kg/day

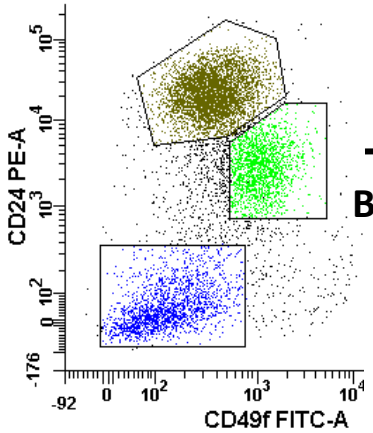
6 wk →

5 → Term. at 6 wk old

5 → Term. at 2 mon old

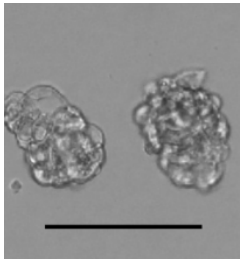
DMBA 30 mg/kg at 2 mon old → 5 → Term. at 4 mon old; 5 → Term. at 4 mon old

→ FACS



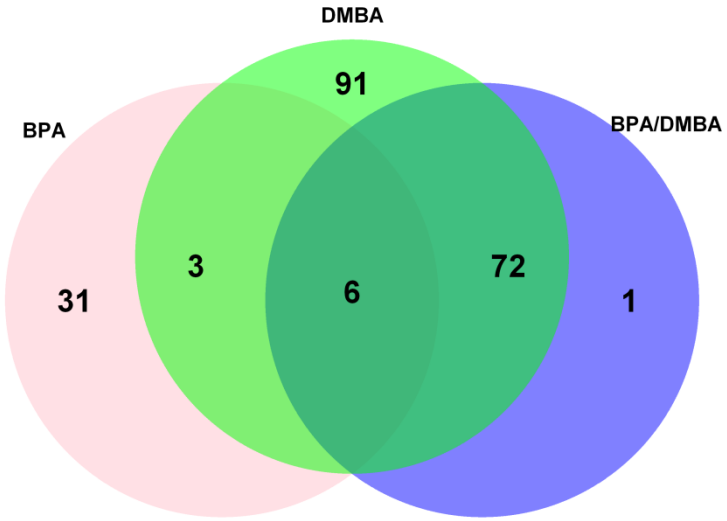
→ Basal

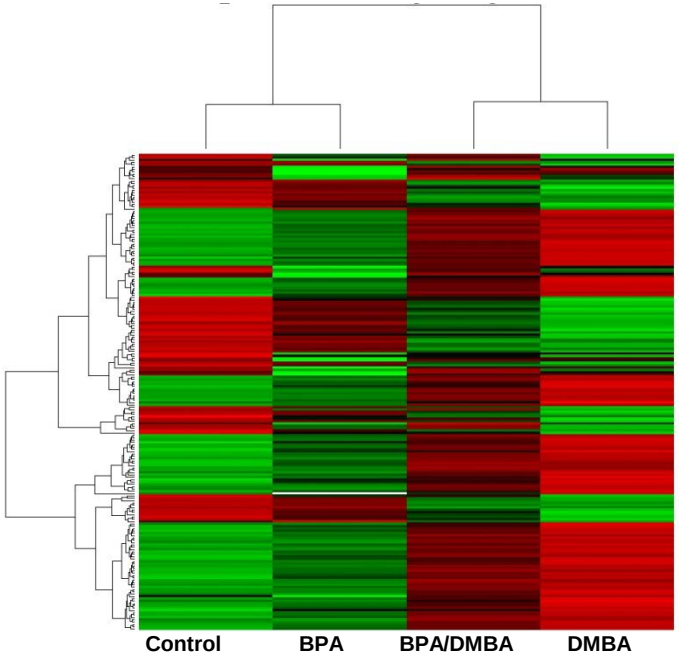
Sphere

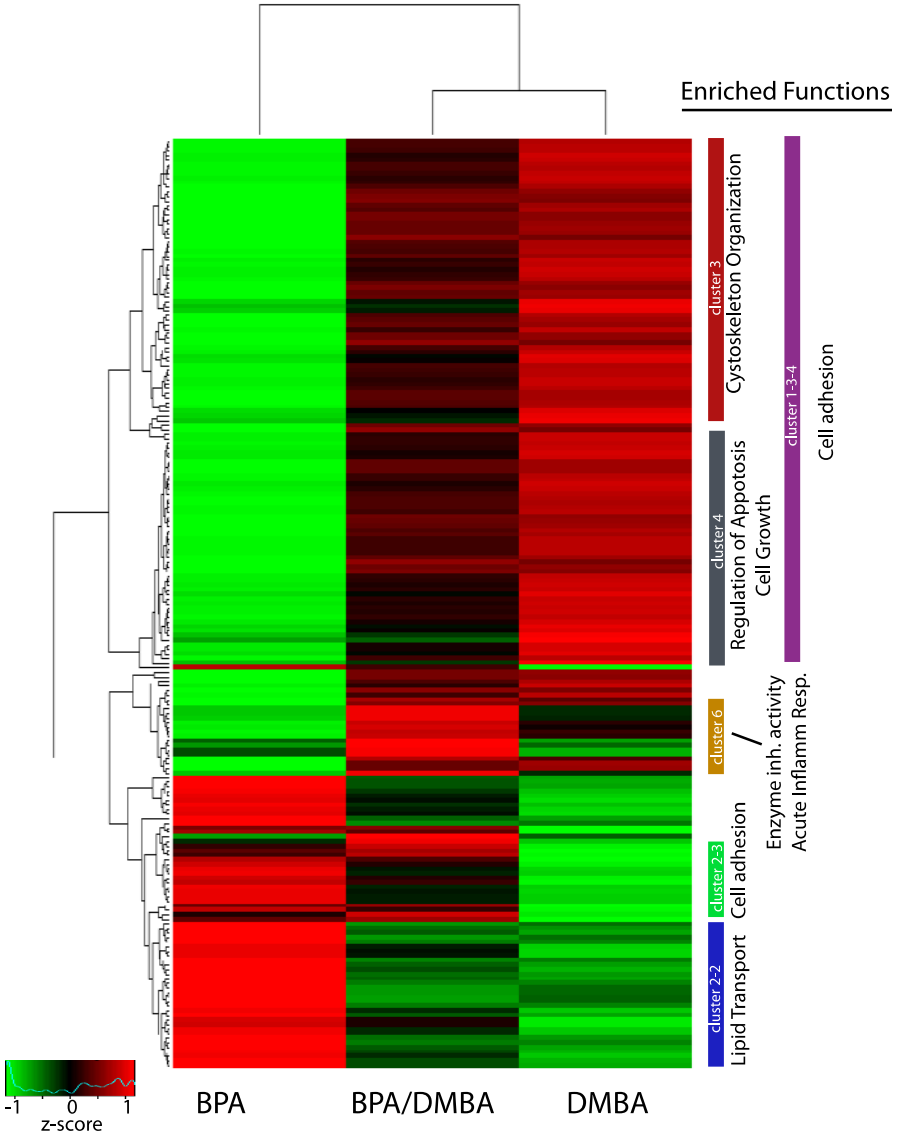


→

Transcriptional Changes







Wang et al., 2014, Cancer Prev Res

Pubertal BPA Exposure Led to Precancerous Gene Signature in MaSC-enriched Spheres

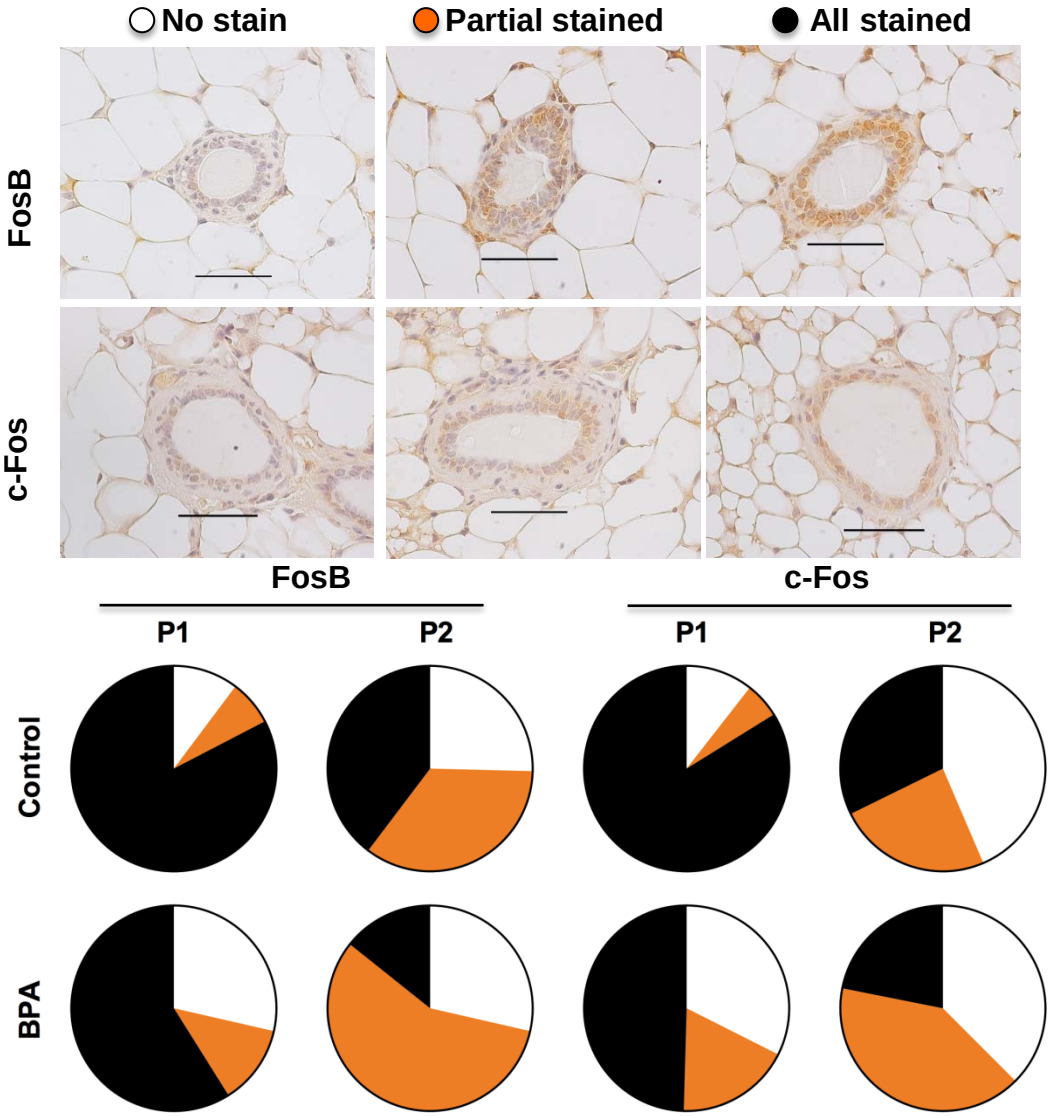
Tripathi A et al 2008: Normal/DCIS tissues in breast cancer patients vs. normal tissues in normal controls

- **Down-regulated**
- **Similar sets of genes**
- **Fosb and Fos**

Symbol	Log2 fold
Fosb	-3.66
Fos	-2.38
Atf3	-1.48
Rgs2	-1.25
Dusp1	-1.03
Nr4a1	-0.92
Egr1	-0.86
Klf4	-0.81
Zfp36	-0.79

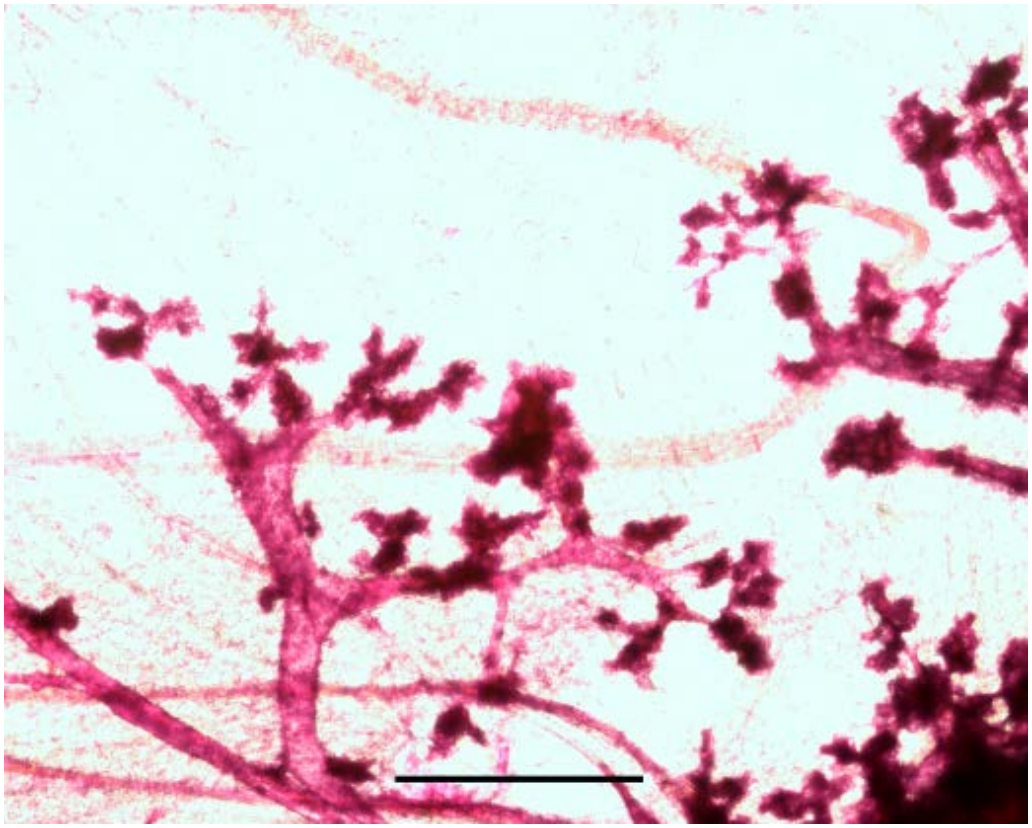
Tripathi A et al.Int J Cancer 2008 Apr 01;122(7):1557-66

Decreased FosB/Fos in Regenerated Glands from BPA treated MaSC



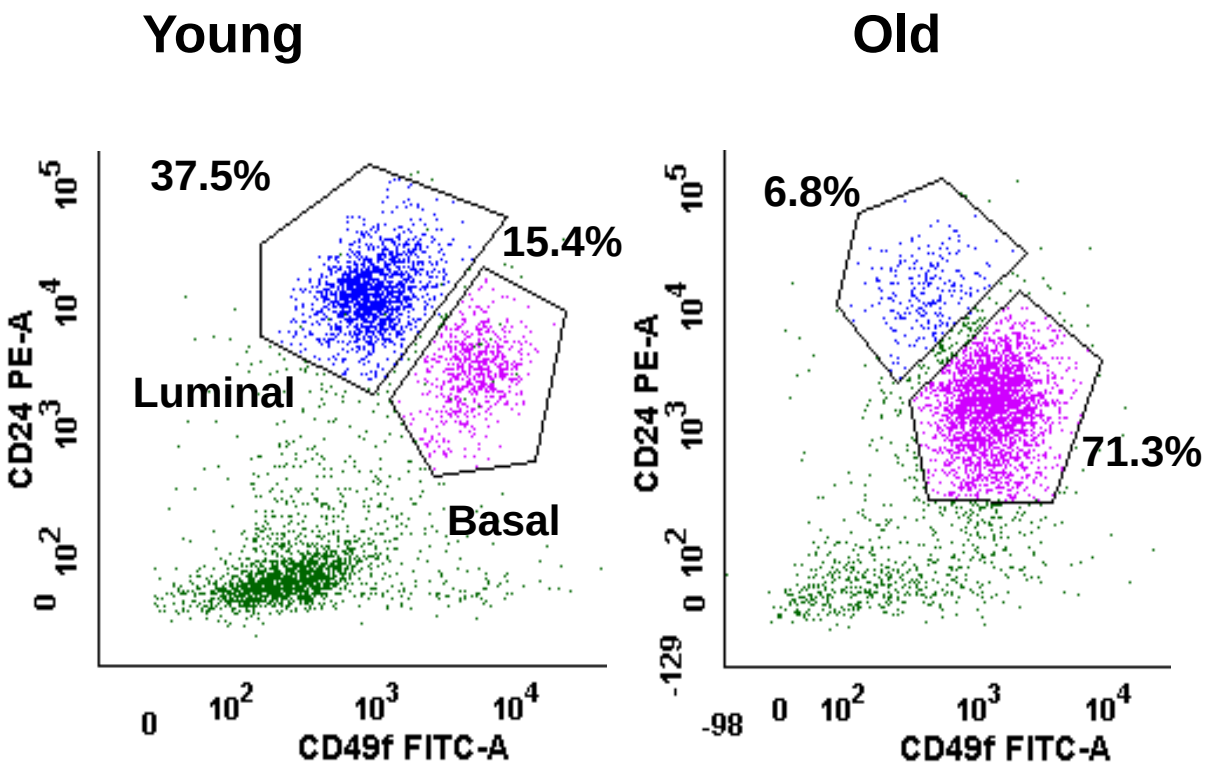
Wang et al., 2014, Cancer Prev Res

The Aged MaSC Story



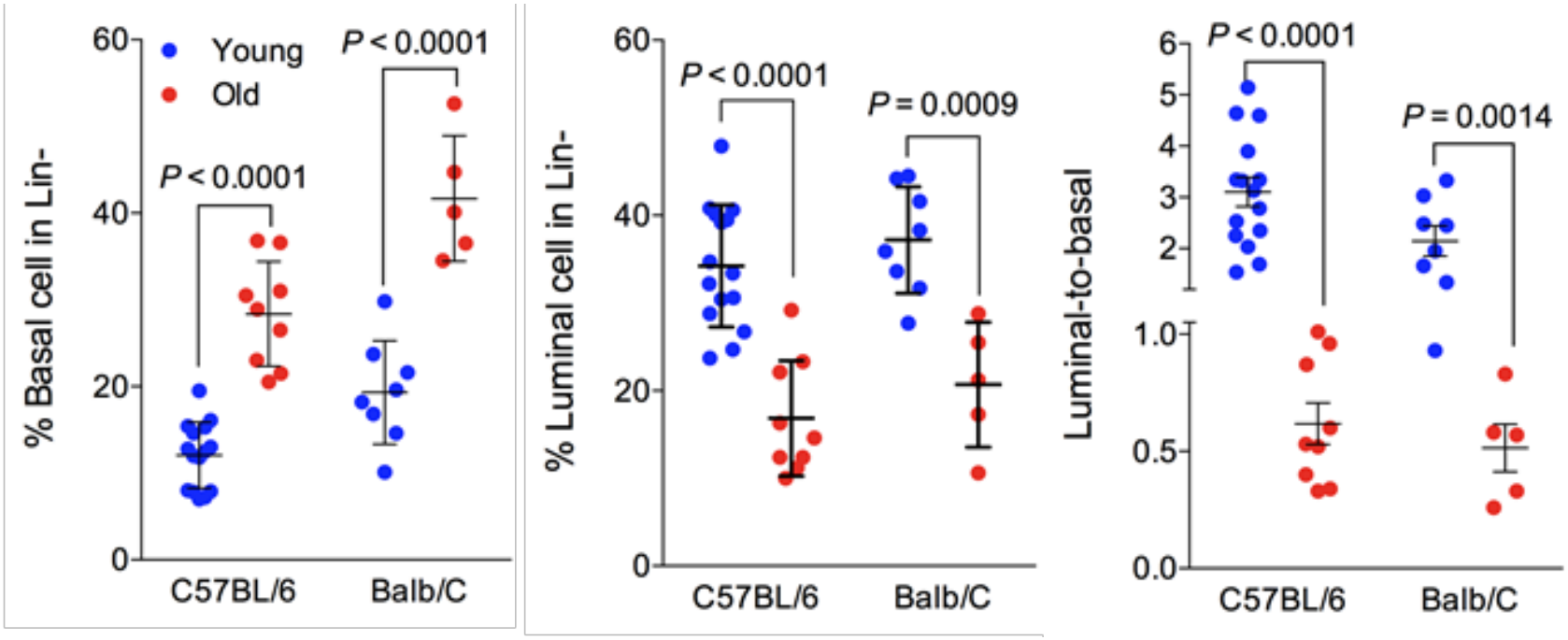
Age is the number one risk factor for breast cancer (5% <40 yr old)

Increased Basal Cell Pool in Old Glands

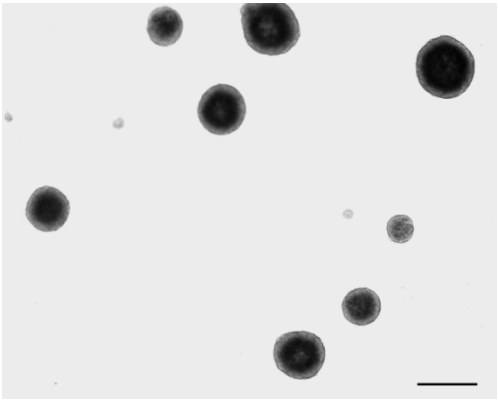


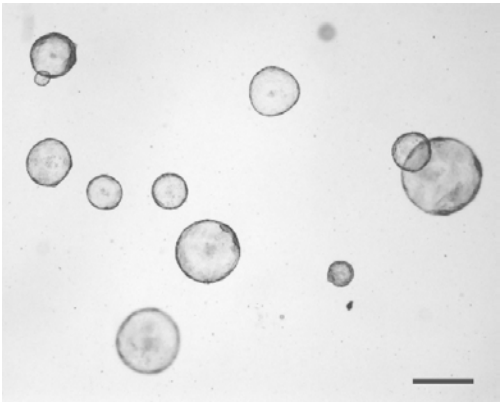
Dong et al., 2016, in review

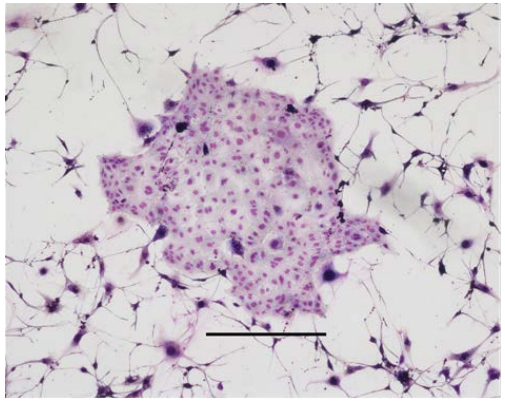
Basal vs. Luminal Cell Pool

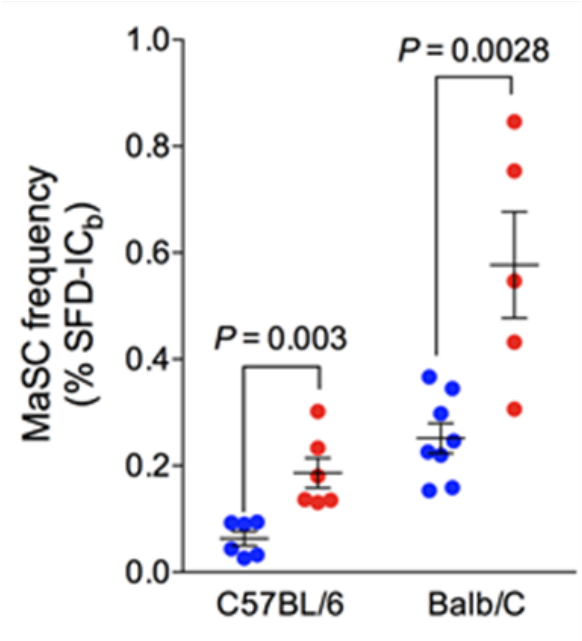


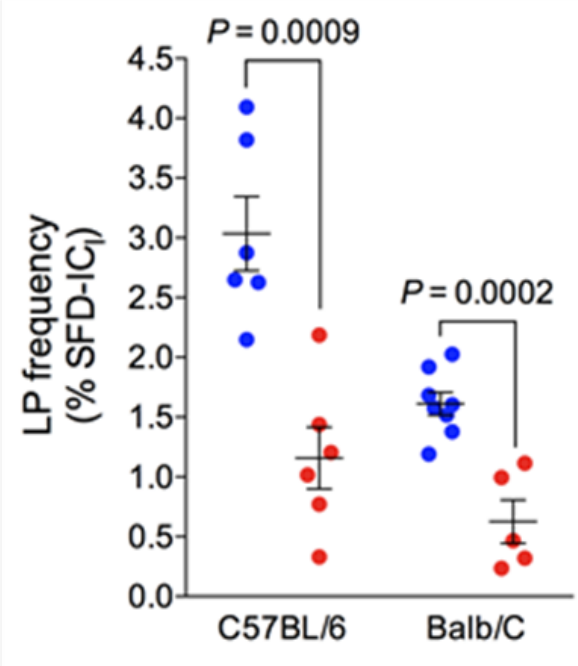
Basal/Luminal Stem Cell Frequency

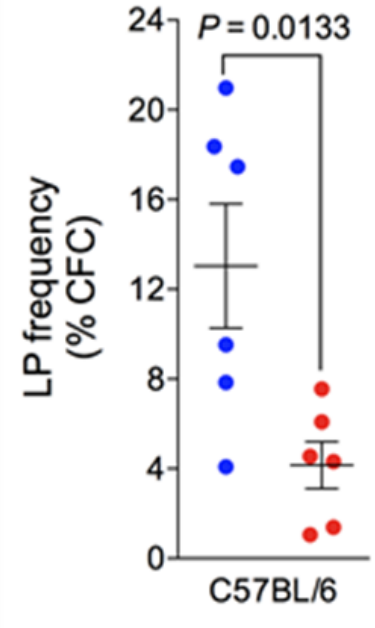




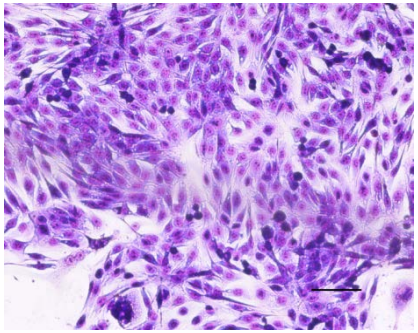


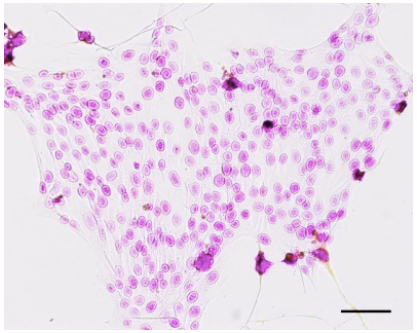


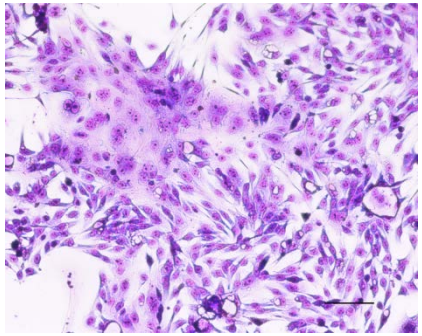


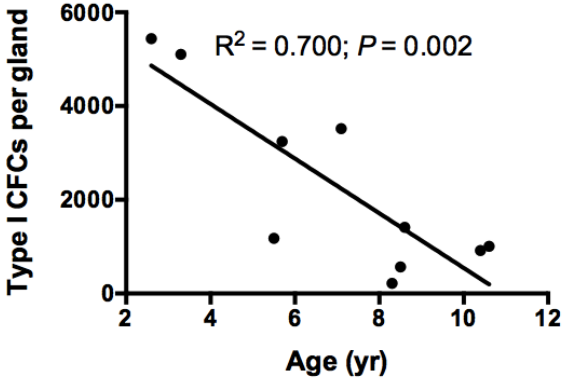


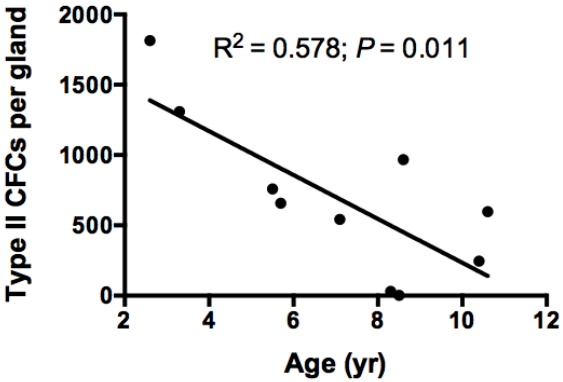
Common Marmosets

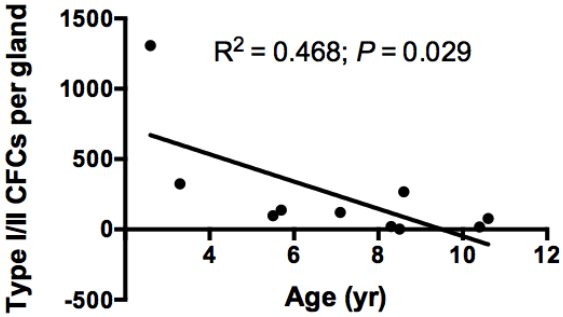


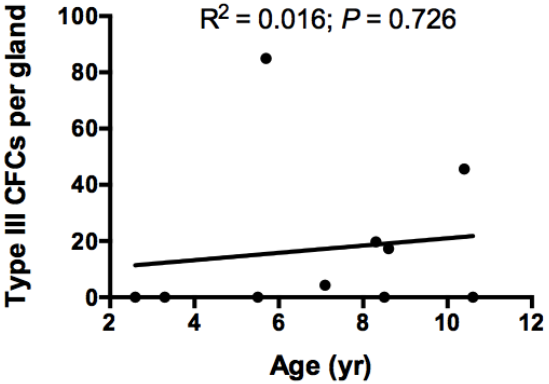


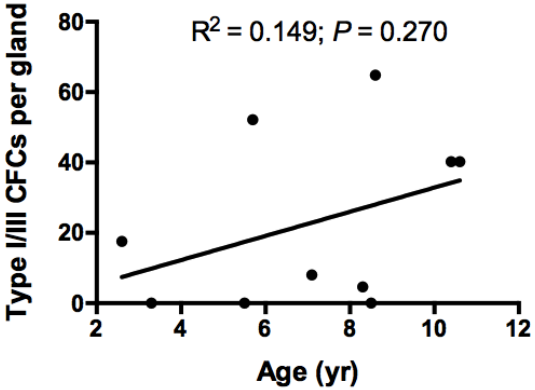


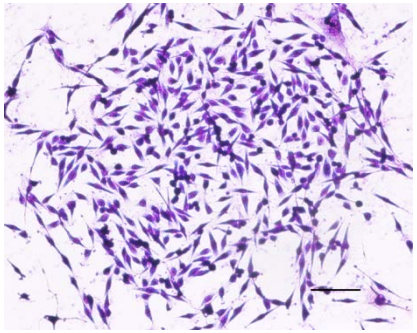








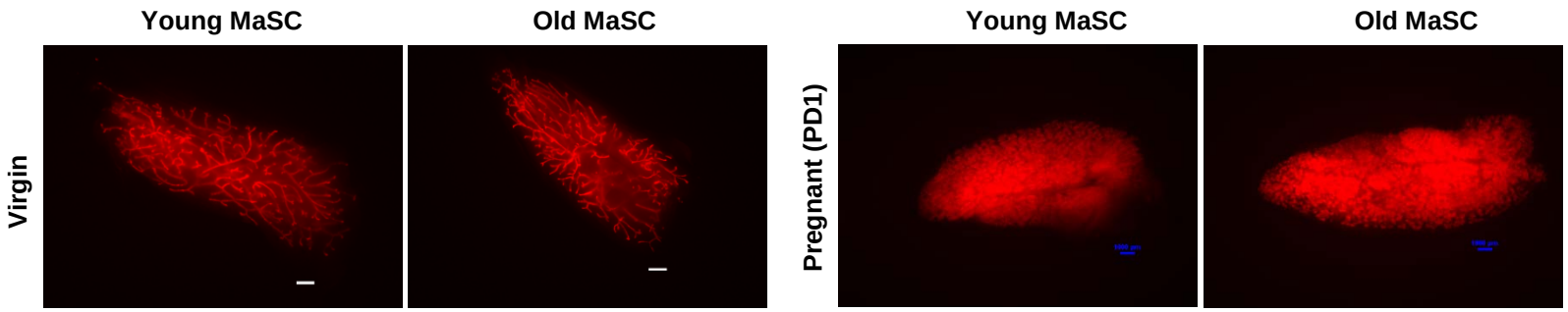




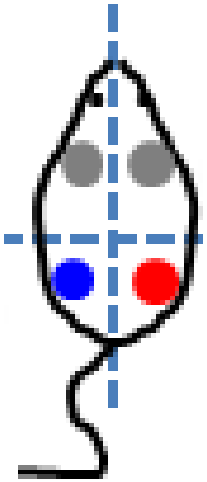
Wu et al., 2016, in revision

BaSC Self-renewal

	Positive/total CFP	
Treatment group	**Young**	**Old**
1st transplants	11/15 (73%)	12/15 (80%)
2nd transplants	5/5 (100%)	5/5 (100%)



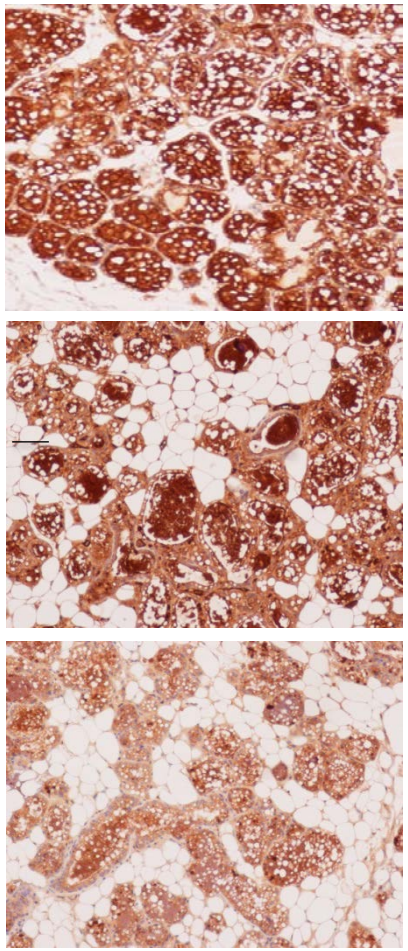
BaSC Differentiation and Function

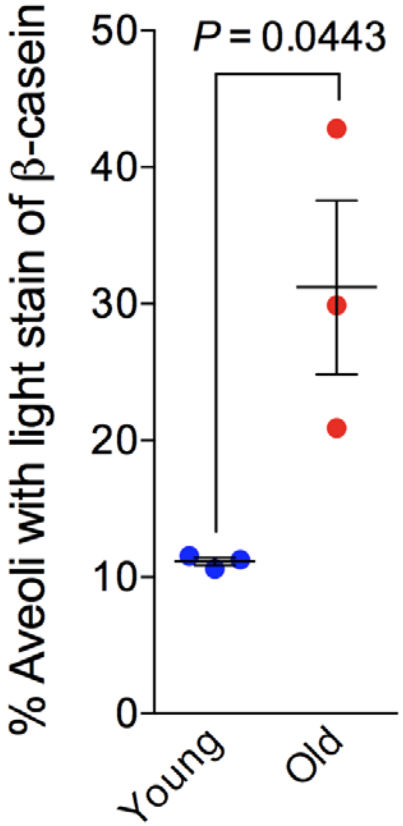


Recipient thoracic

Young donor

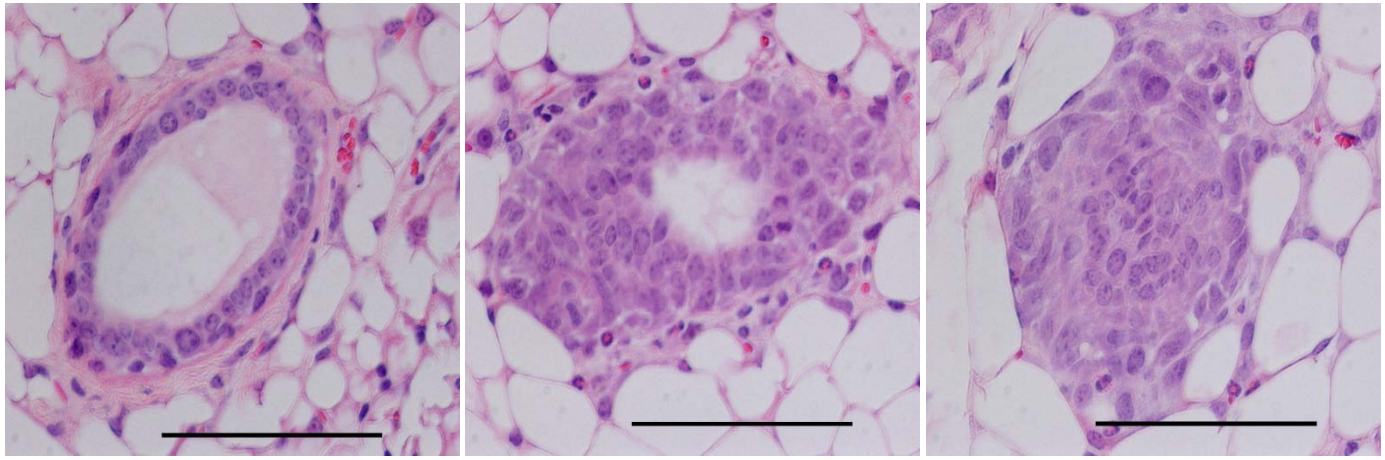
Old donor



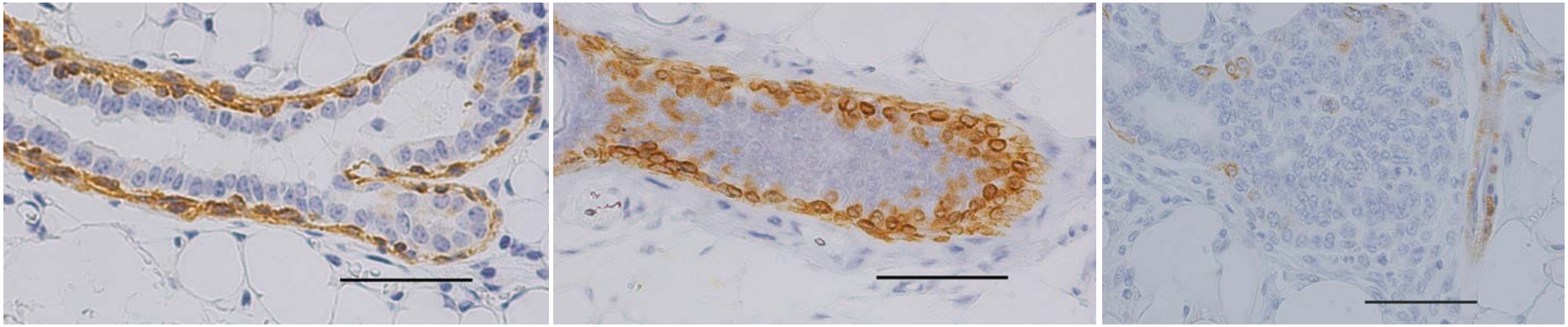


Early Neoplastic Lesions

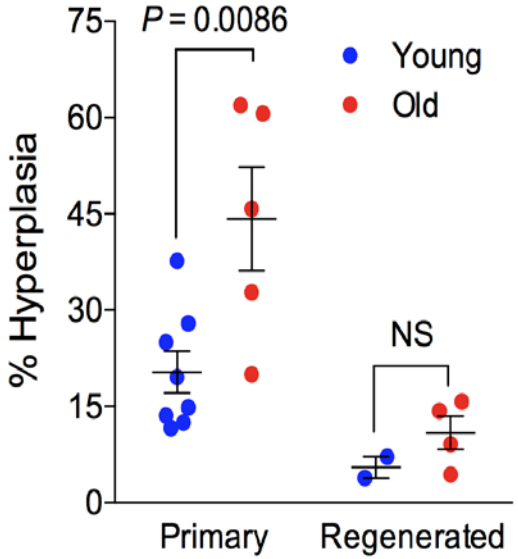
Normal **Hyperplasia** **ADH/DCIS**



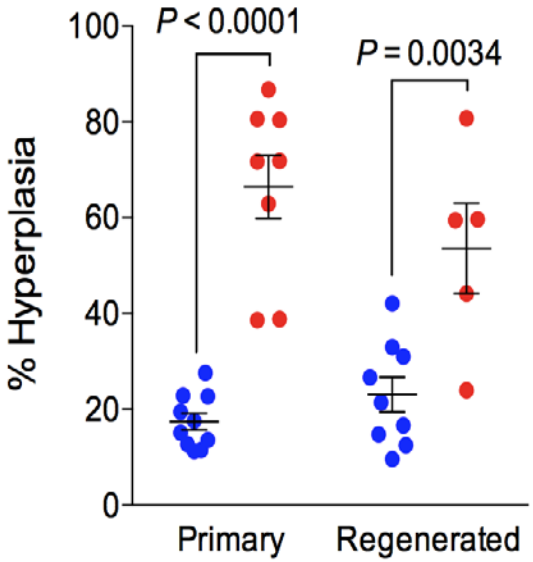
CK5 staining



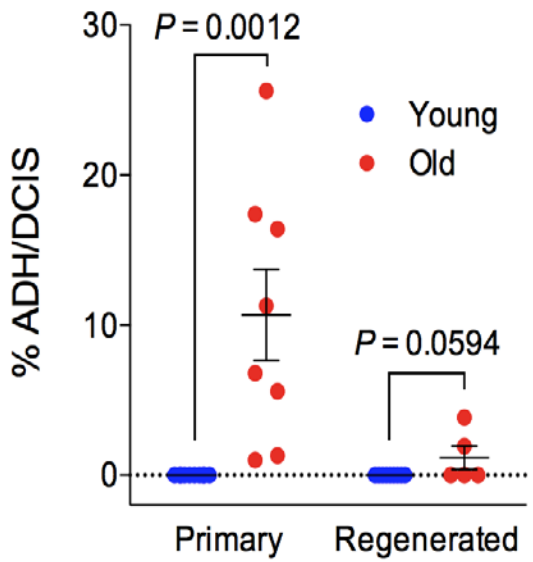
Regenerated Glands-Neoplastic Lesions



DsRed-C57BL/6



Balb/C



Balb/C

Summary

- Increased basal SC and decreased luminal SC frequency in aged glands
- Decreased self-renewal in old basal SCs
- Dysfunction of differentiation (milk production) in old basal SCs
- Increased transformation potential in old basal MaSCs

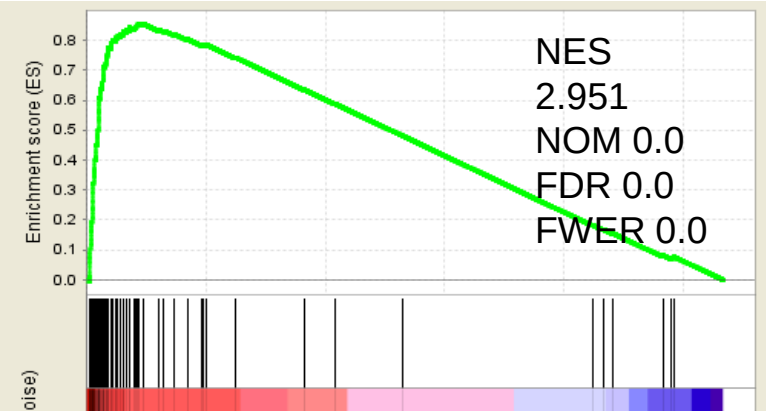
Q1: Why the basal cell compartment expanded?

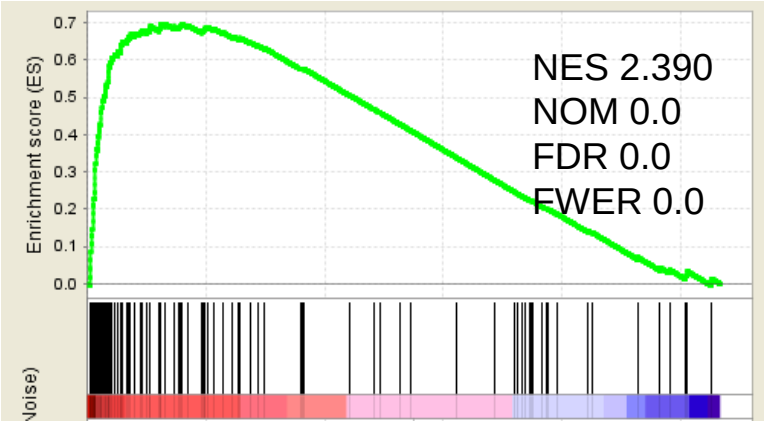
Q2: What's the underlying mechanism?

GSE analysis: Enriched Luminal Signature in Old Basal Cells

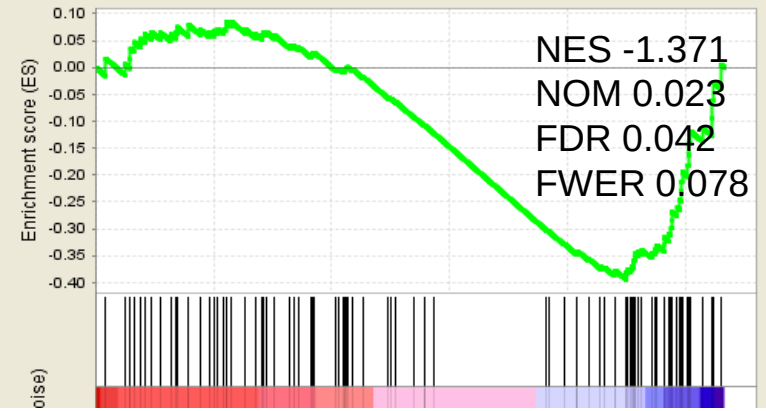
Ours | **Lim et al. 2010**

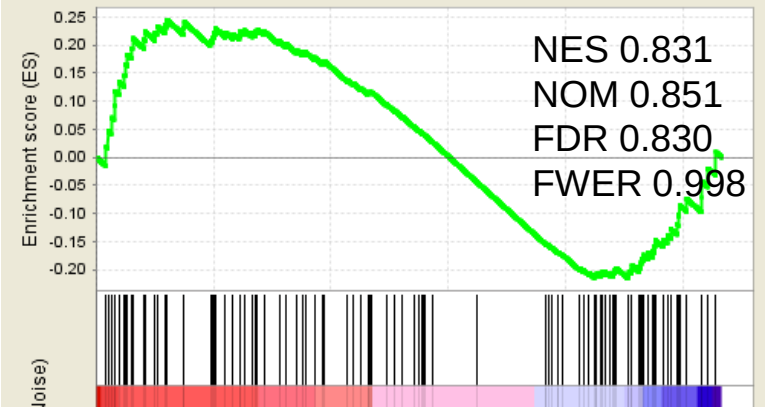
Basal cells





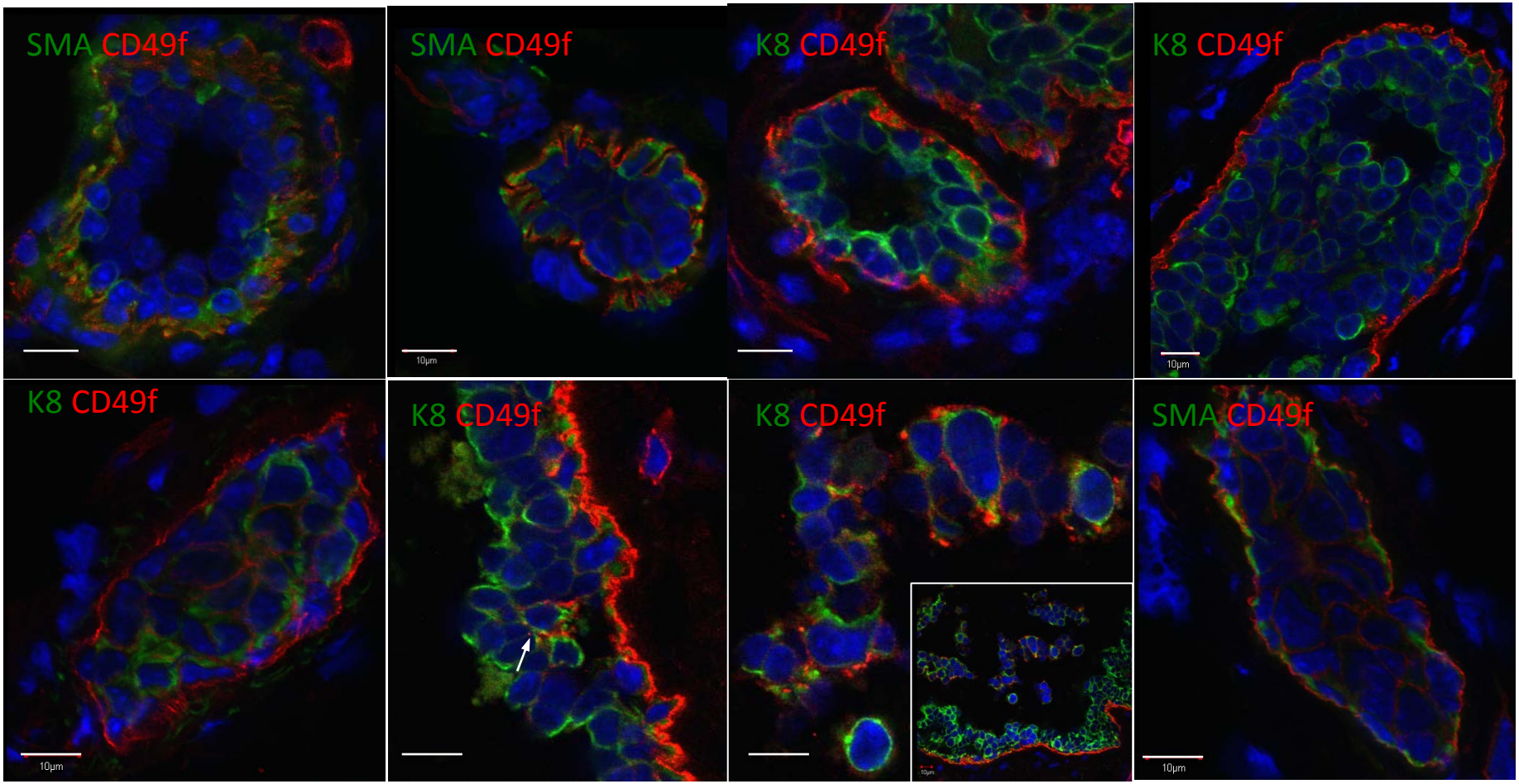
Luminal cells



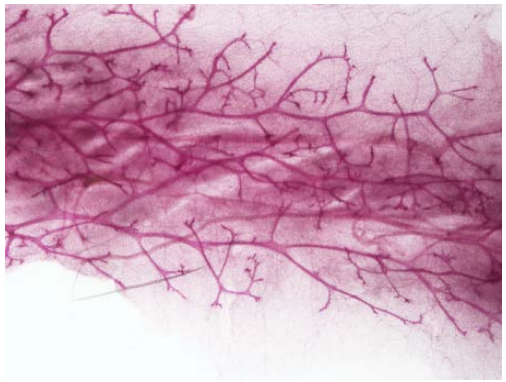


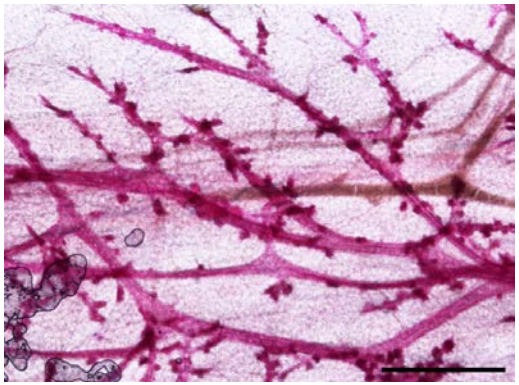
Genes Highly Expressed in Old **Genes Highly Expressed in Young**

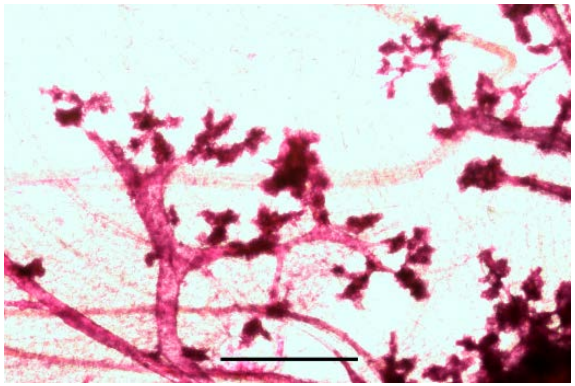
K8+ Luminal Cells Expressing High CD49f



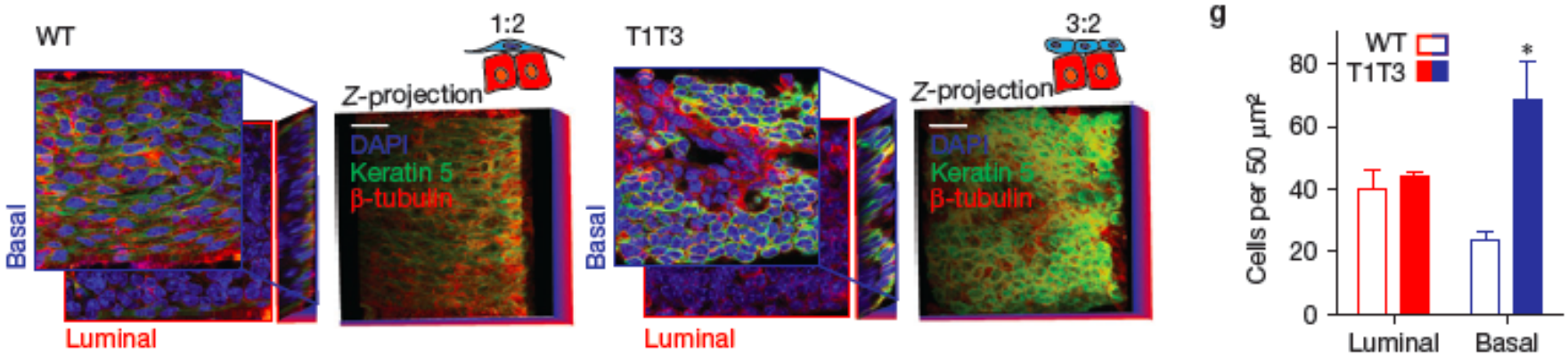
Expanded CD49f hi Basal-like Cells





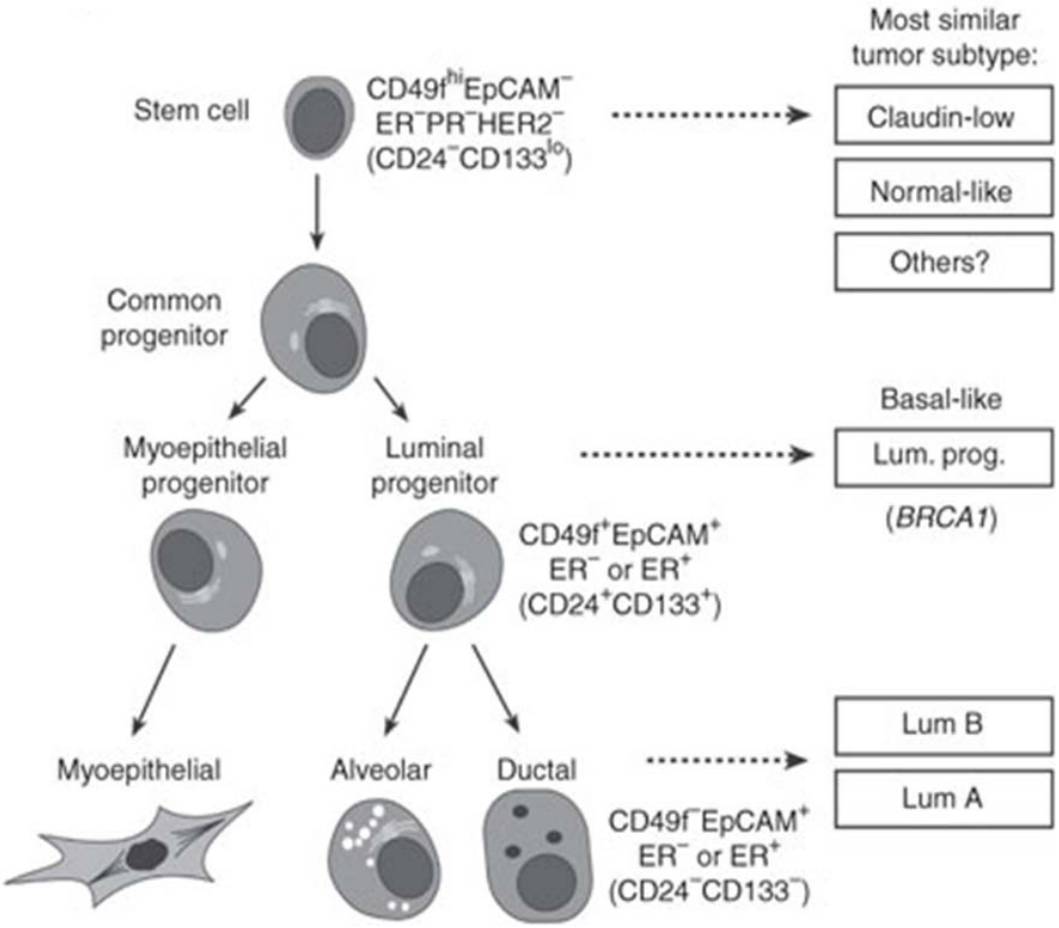


- ❖ Alveolar basal cells
- ❖ K8 positive luminal cells
- ❖ Ductal basal cells



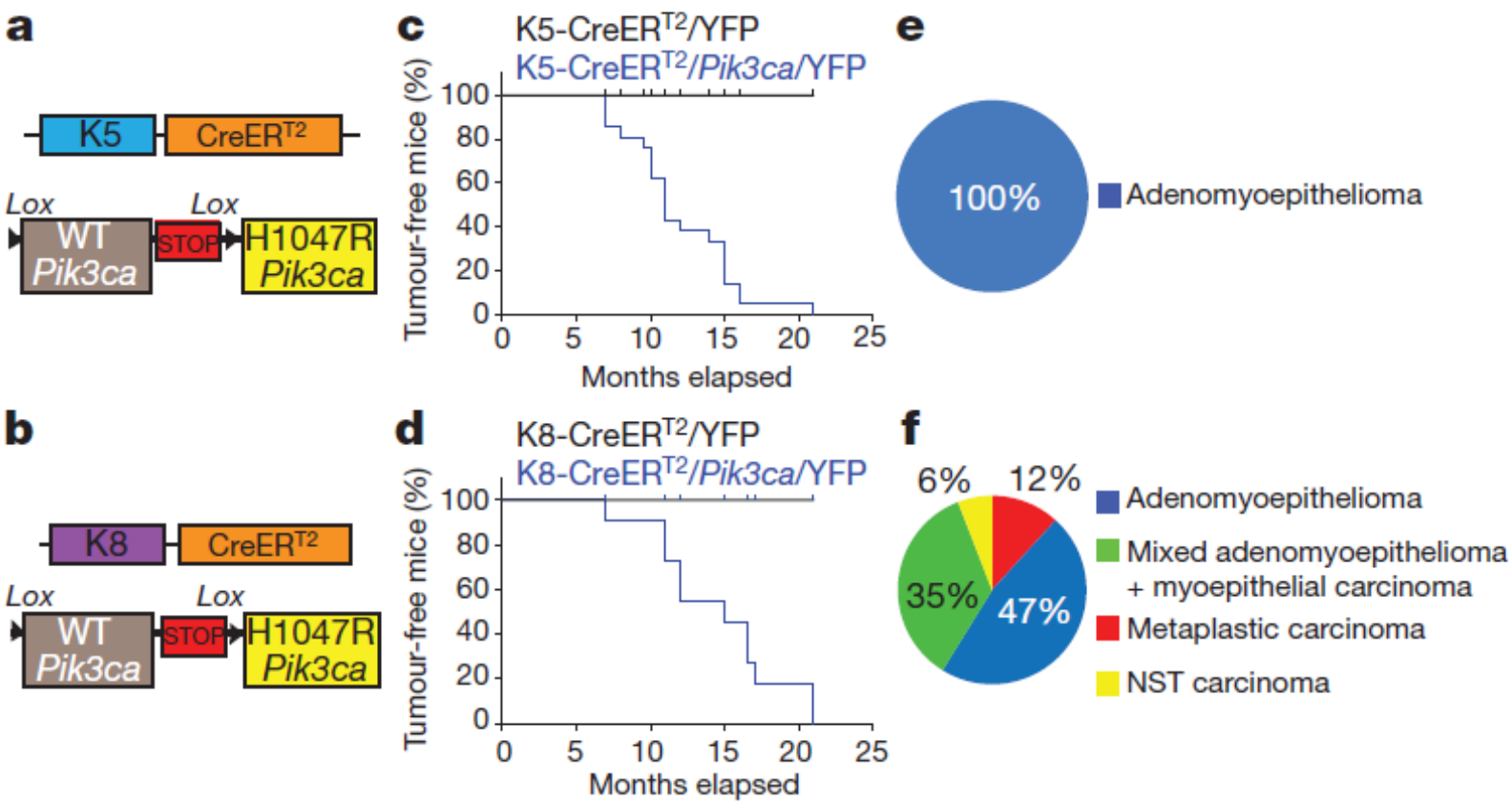
Jackson et al., 2015 Nature Cell Biology

Gene Association Analysis



Lim et al., 2009, Nat Med 15:907-13

Oncogenic Induction in vivo

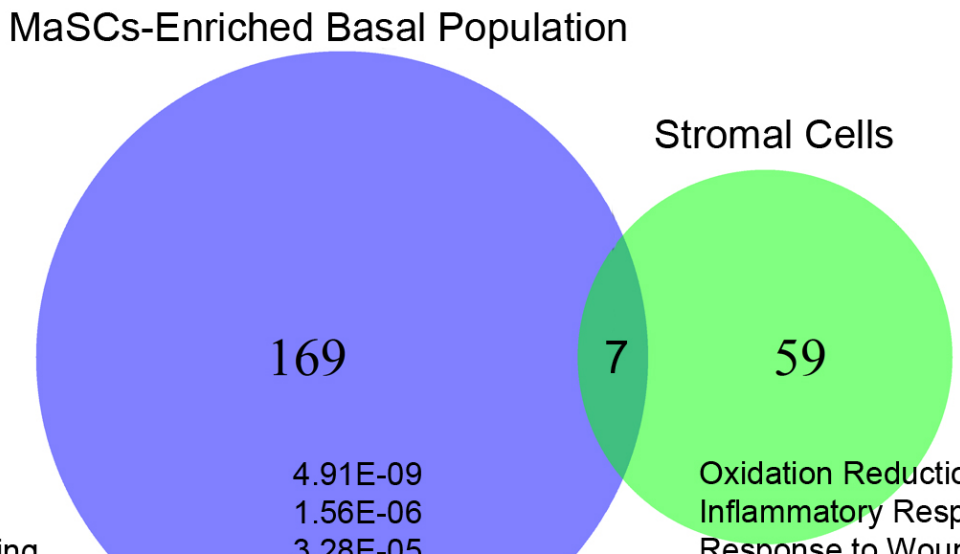


Van Keymeulen et al., 2015, Nature

Model	Oncogene	Tumorigenesis	Reference
Isolated cells (EpCAM$^-$CD49f$^+$)	KRAS, PIK3CA, TP53 or KRAS	Normal-like (ER+)	Nguyen et al., 2015

Nguyen et al., 2015, Nature

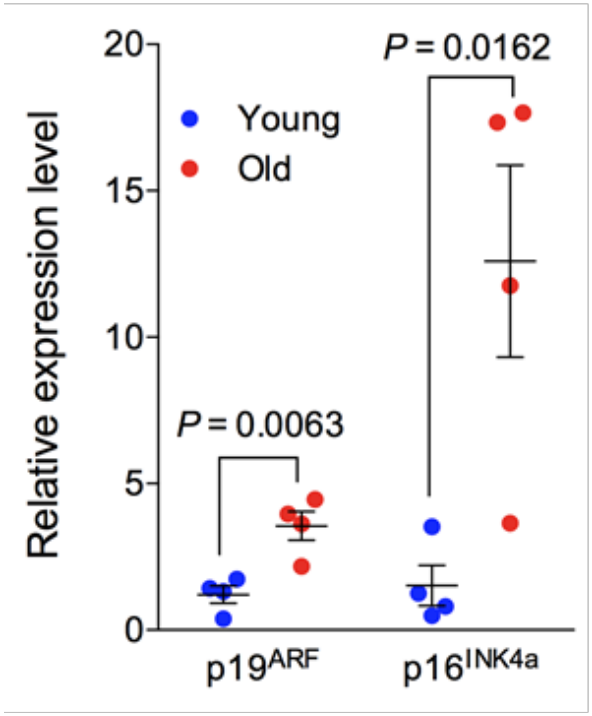
Increased immune/inflammation signals (MaSC and Niche)

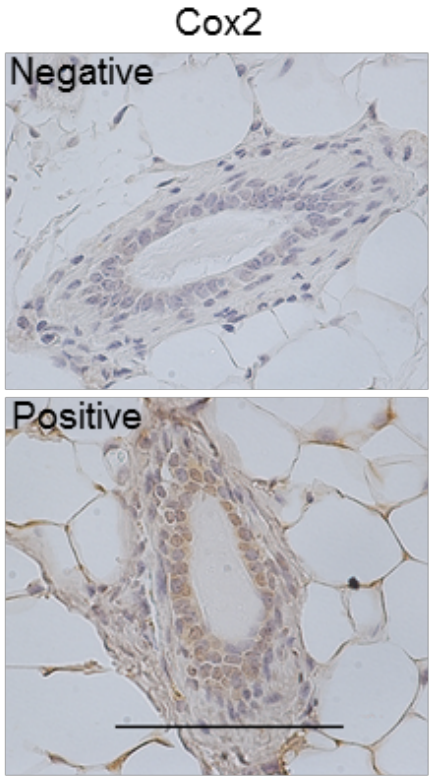


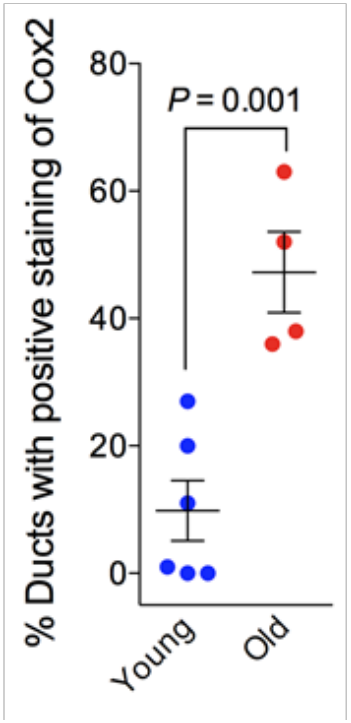
Immune Response	4.91E-09
Defense Response	1.56E-06
Response to Wounding	3.28E-05
Cell Adhesion	1.07E-04
Biological Adhesion	1.09E-04
Epithelium Development	3.11E-04
Inflammatory Response	3.47E-04
Innate Immune Response	6.08E-04
Acute Inflammatory Response	1.14E-03
Negative Regulation of Cell Proliferation	6.24E-03

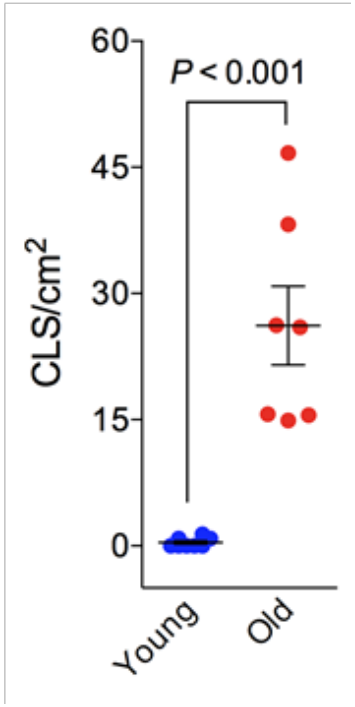
Oxidation Reduction	1.72E-04
Inflammatory Response	6.15E-04
Response to Wounding	6.41E-04
Pyruvate Metabolic Process	2.67E-03
Defense Response	1.19E-02
Immune Response	1.45E-02
Hormone Catabolic Process	1.54E-02
Positive Regulation of Response to Stimulus	1.96E-02
Acute Inflammatory Response	2.59E-02
Fatty Acid Biosynthetic Process	2.59E-02

Inflammation Signal in MaSC Niche

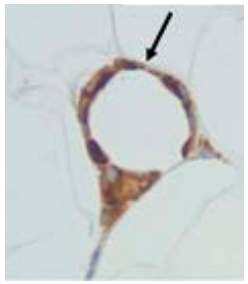




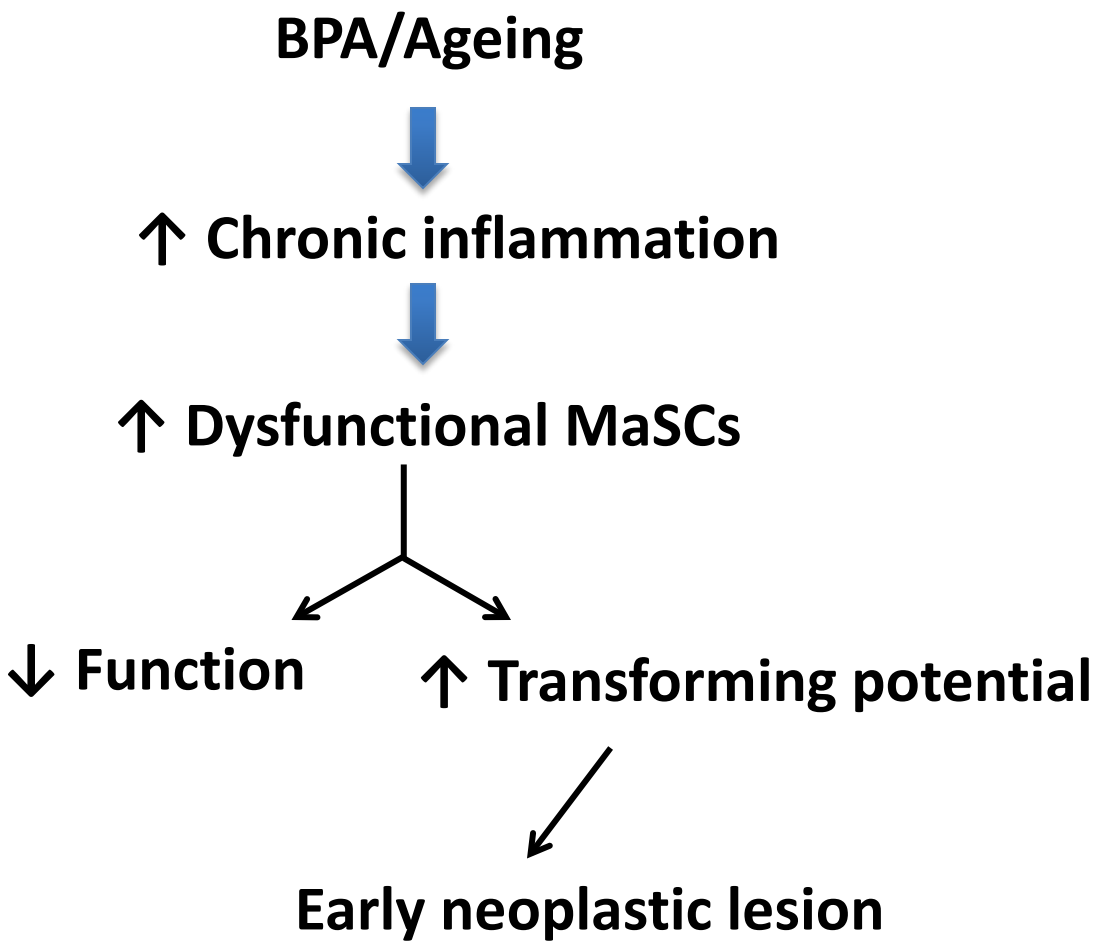




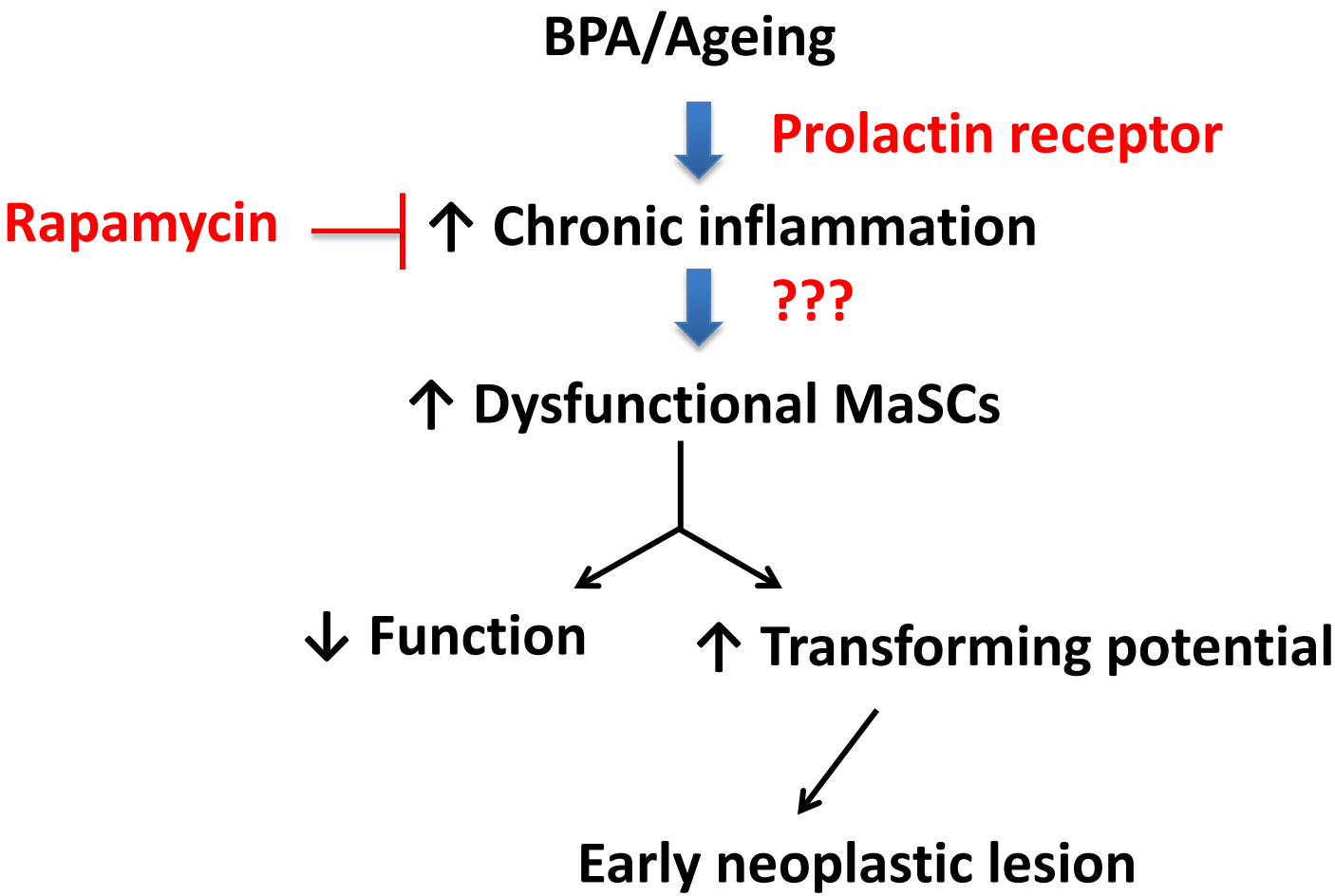
Crown-like Structure



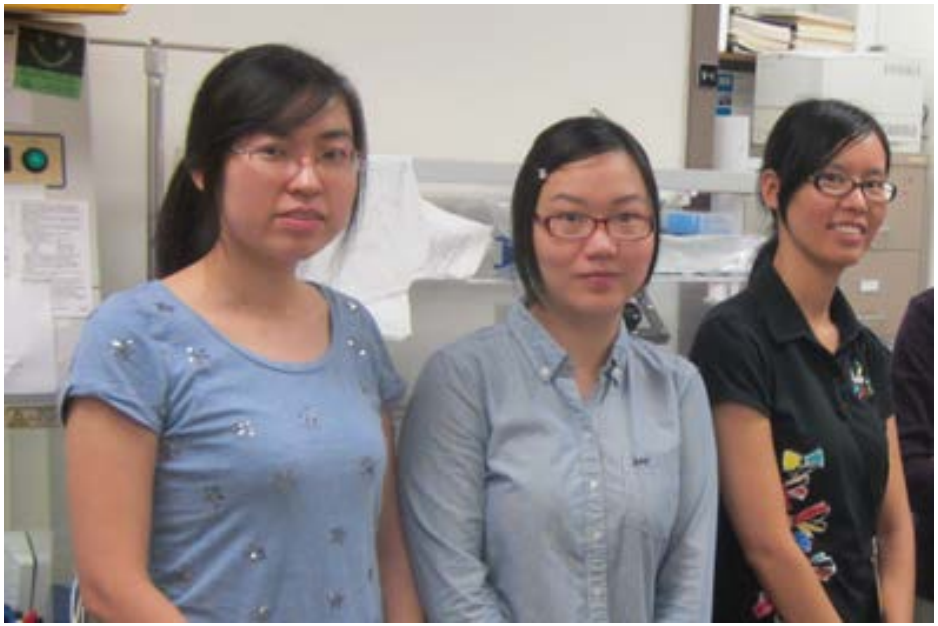
Current Working Model

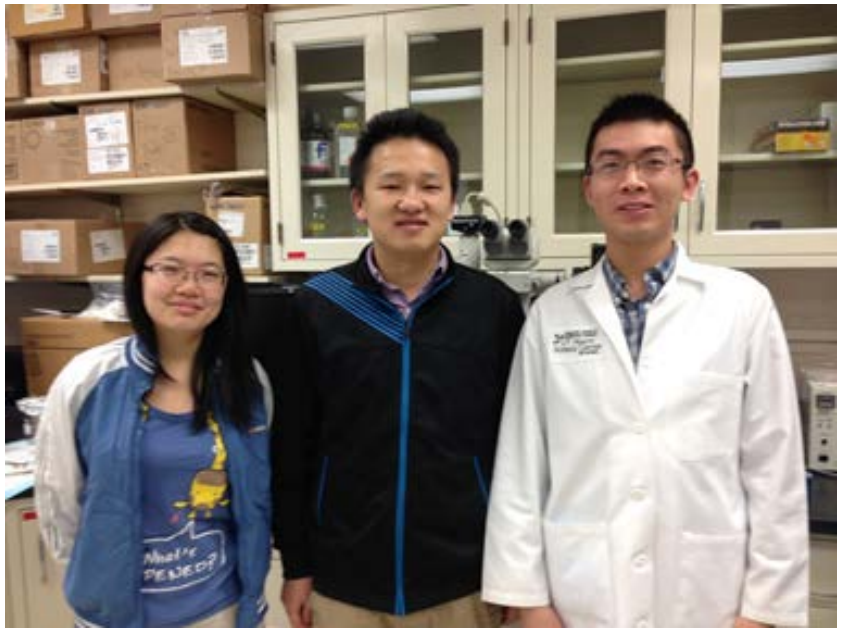


Current Working Model



Acknowledgements





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- National Natural Science Foundation of China

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