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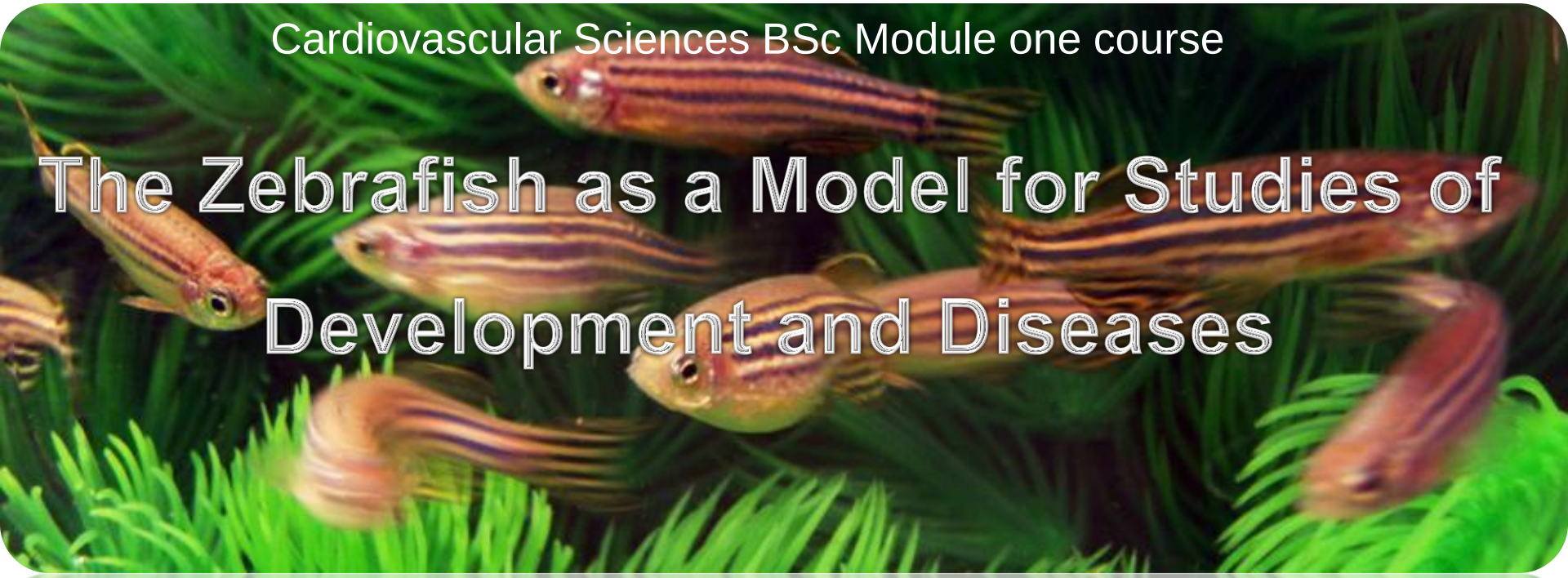
Cardiovascular Sciences BSc Module one course

The Zebrafish as a Model for Studies of Development and Diseases



Kar Lai Poon, Ph.D.

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Lecture Outline

Zebrafish as a model for studying development and diseases

- Introduction
- Limitations
- Comparative anatomy
- Zebrafish development
- Zebrafish Heart and CCS Development
- Advantages
- Disease model : Cardiac Arrhythmias and Cardiomyopathy
- Zebrafish heart regeneration

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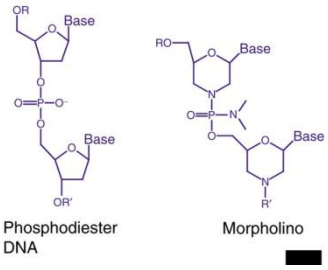
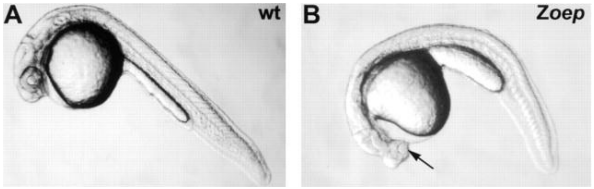
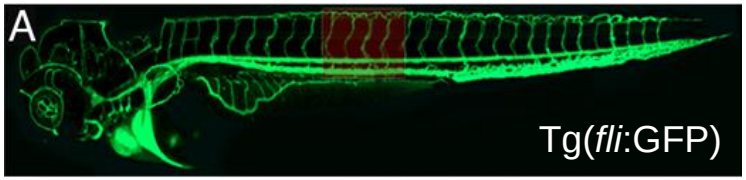
Introduction to Zebrafish

- *Danio rerio,* a tropical freshwater fish, cyprinidae family
- Initially used as a model organism for studies of early vertebrate development
- 1960s – George Stresinger obtained zebrafish to work on
- 1990 – first international zebrafish conference
- 1996 – mutagenesis screen identifying hundreds of mutants published
- 2000s - Zebrafish genome sequenced
  - Morpholino technology for gene knockdown introduced
  - Transgenic lines generated
  - 'validation' phase
- Now, for modeling human diseases, drug discovery, environmental monitoring etc







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Limitations of Zebrafish Research

- Evolutionarily more distant from humans than mammals, findings from fish experiments will likely have to be replicated in mammals before being directly correlated to human therapy
- Some organs/cell types are too small, require specialized microscopes /technologies and can be technically challenging to work with
- Reverse genetics are not as well developed as mouse, lack ES cells and homologous recombination.
- Lack of certain mammalian/ human structures/characteristics for e.g, without septum
- Lives in the water, difference in physiology
- Limited resources e.g, number of cross-reacting antibodies
- Modest costs associated with starting a zebrafish facility.

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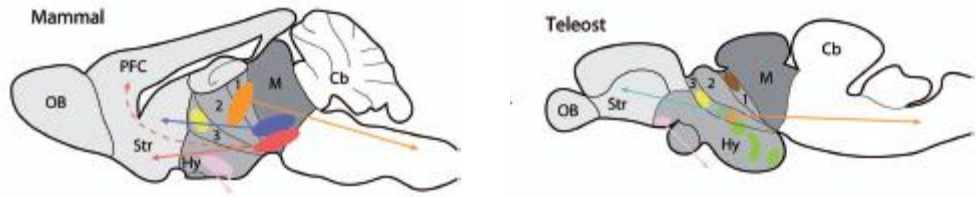
Comparative Anatomy Zebrafish vs Man

Brain

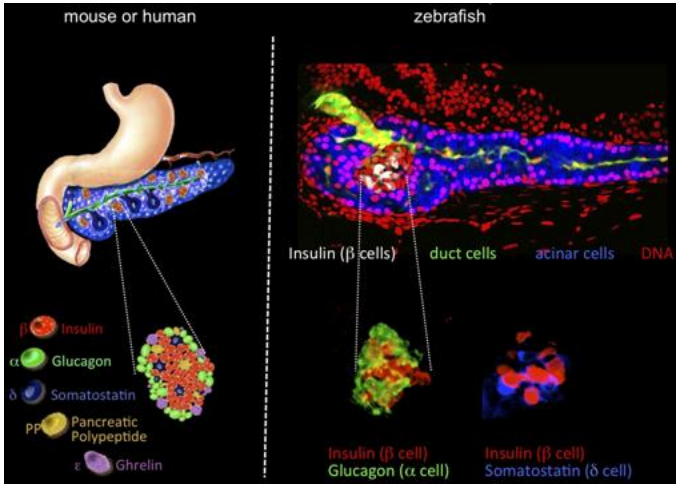
Enlarged and well developed forebrain that confers cognitive function



Primitive brain : small forebrain and big hindbrains

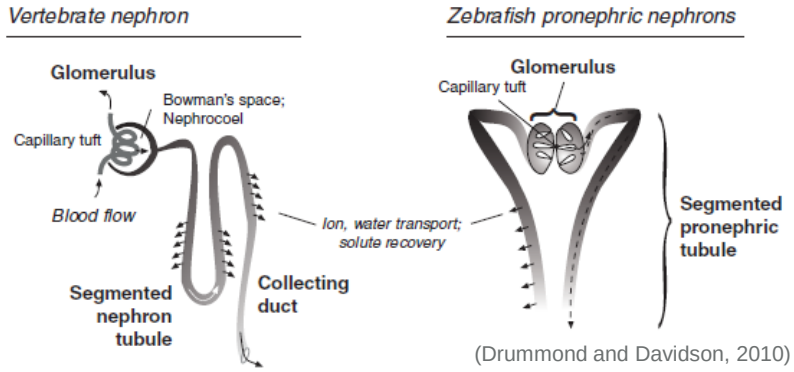
(Yamamoto and Vernier, 2011)

Pancreas



Zebrafish endocrine islets tightly embedded inside the exocrine tissue to form a pancreas quite like a mammalian pancreas

Kidney



(Drummond and Davidson, 2010)

Nephron shape, function, cell types and gene expression are remarkably conserved.

relative simplicity and stereotyped organ development

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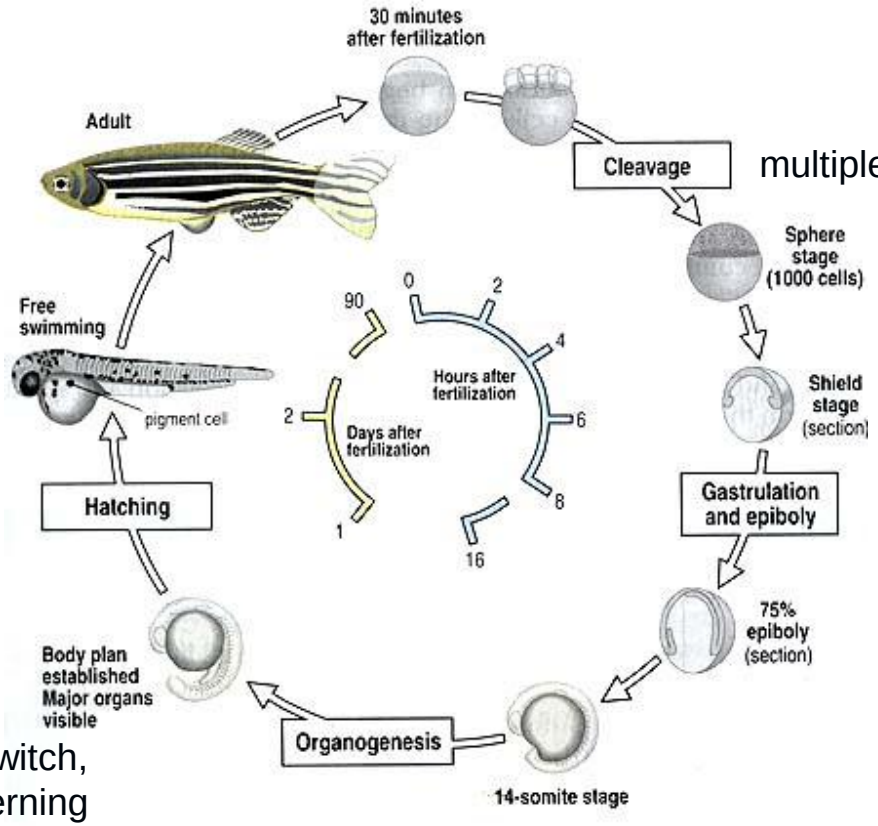
Zebrafish Development

At 3-4 months of age, sexual maturity is reached.

Growth, morphogenesis and maturation of organs

Heart contracts blood circulate

Embryos begin to twitch, brain patterning forming heart field morphogenesis



multiple cell division

Mid blastula transition (MBT) when transcription of zygotic genome begins

Formation of 3 germ layers: ectoderm, mesoderm and endoderm
via Epiboly which involves complex cell migration

First wave of neurogenesis
Somitogenesis etc

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Zebrafish Heart Development

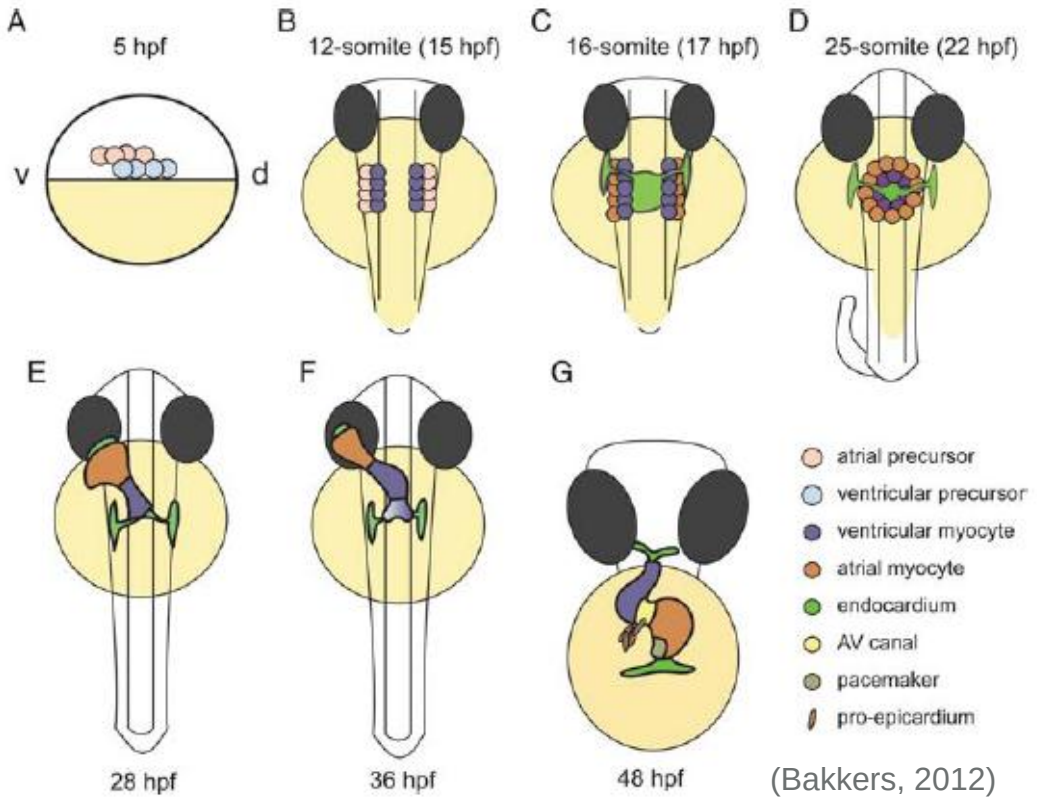
At 5 hpf, distinct precardiac cells are specified and located at the lateral marginal zone.

During gastrulation, these precardiac cells move towards the mid-line.

At 12-somite stage, ventricle progenitor cells reached the anterior later plate mesoderm (ALPM) where cardiogenic differentiation is initiated shown by expression of cardiac myosins.

During mid- and late-somite stages, myocardial tissue expands by continuous cardiogenic differentiation.

Endocardial cells migrated from ALPM towards mid-line prior to the myocardial cells. Bilateral heart fields fuse at the mid-line forming a cardiac disc.



(Bakkers, 2012)

Cardiac morphogenesis transforms the cardiac disc into a cardiac tube with inner lining of endocardium.

At 22 hpf, the linear heart tube has formed.

At 36 hpf, cardiac looping takes place, and the constriction at the position of the AV canal is first visible.

Ellipsoid extracardiac pro-epicardial cells located near the AV canal start to cover the myocardium with an epicardial layer.

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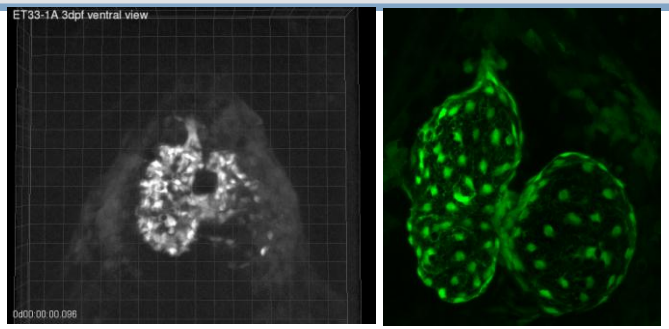
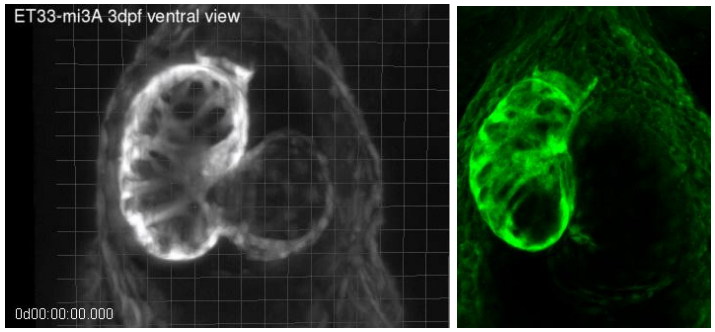
Zebrafish Heart Development

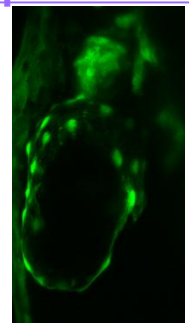
Endocardium



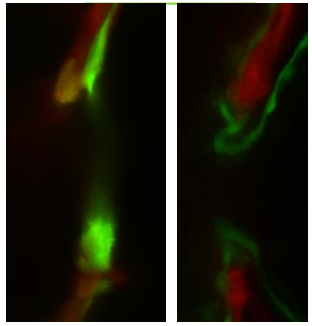
Myocardium

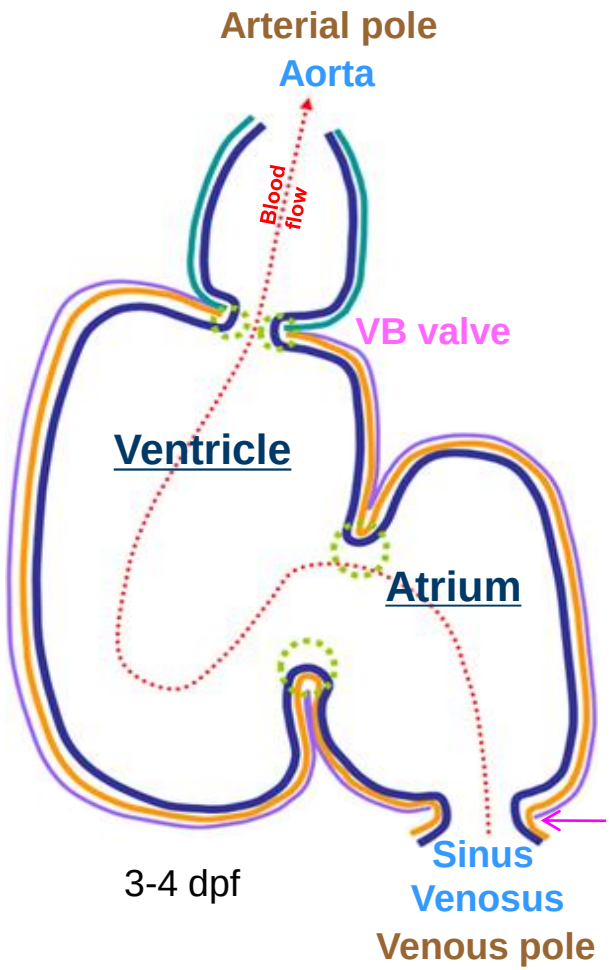


Epicardium

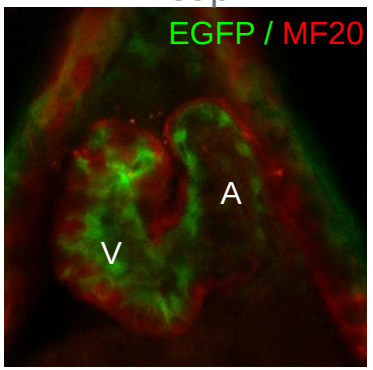


AV valve

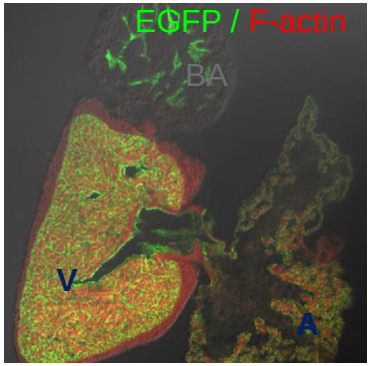




3dpf



Adult heart section



MF20, F-actin : myocardial marker

(Poon et al., 2010)

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Conserved Cardiovascular Developmental Events

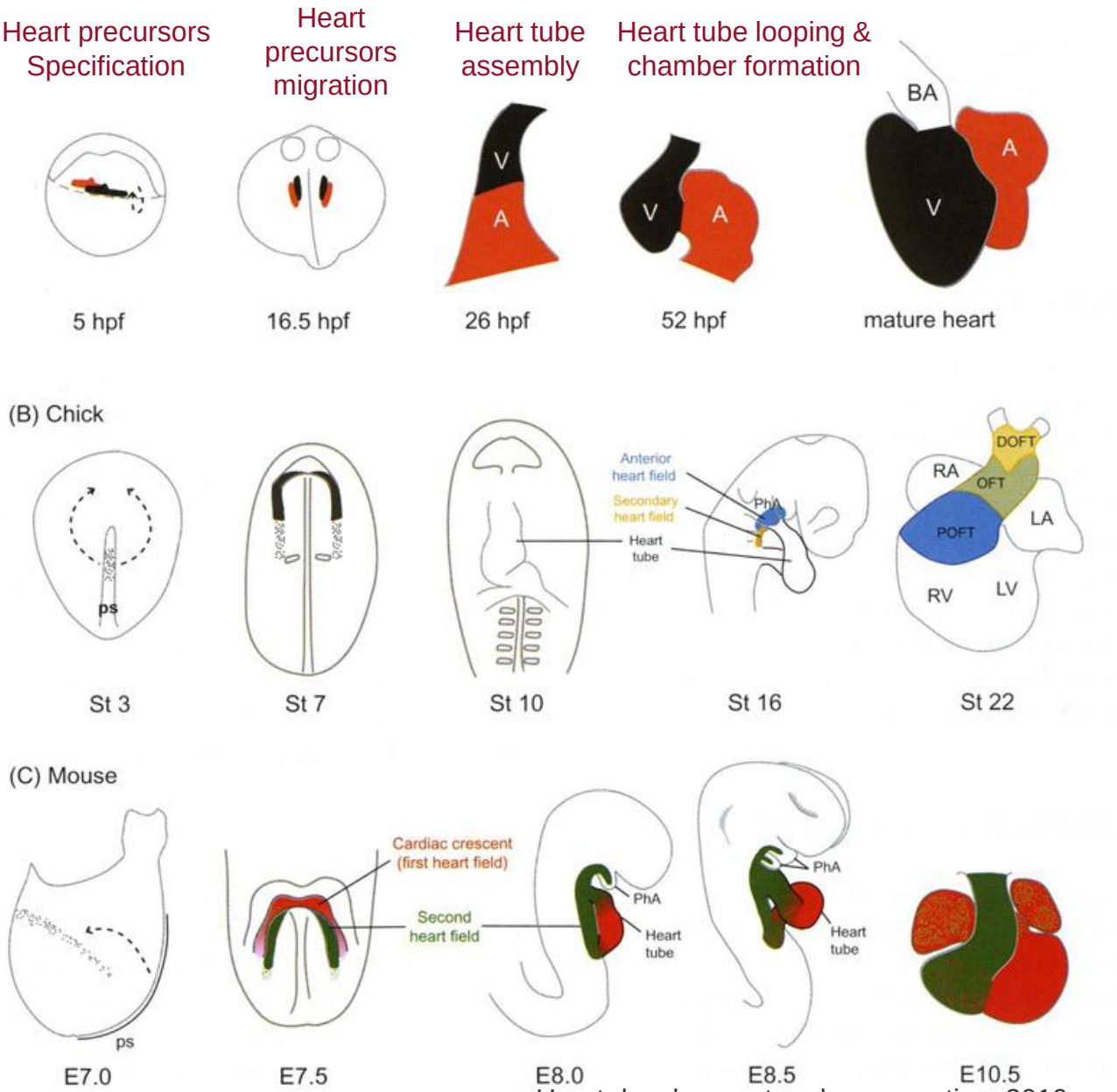
Table 1. Milestones of early heart development in different species

	mouse	chick	human	frog	zebrafish
Migration of precardiac cells from epiblast	7 dpc (primitive streak)	HH4 (definitive streak)	15-16 days	stage 10	50% epiboly (5.5 hpf)
First evident assembly of myocardial plate	7 dpc (late primitive streak; just presomite)	HH5 head process (19-22hrs)	18 days	~stage 13	8-10 somites (~13 hpf)
Generation of single heart tube initiated	8 dpc (5-10 somites)	HH9 (7 somites)	22 days (4-10 somites)	stage 28	20 somites (ring) (~19 hpf)
Tubular heart starts contraction	8.5 dpc 8-10 somites)	HH10 10 somites (33-38 hrs)	23 days	~stage 33	26 somites (22 hpf)
Looping	8.5 dpc	HH11 (11-13 somites)	23 days	stage 33-36	33 hpf
Cushions form	9.5 dpc	HH17	28 days (30-38 somites	~stage 41	48 hpf

The mouse data are primarily from DeRuiter et al. (1992) and Kaufman and Navaratnam (1981), the chick from DeHaan (1965), Garcia-Martinez and Schoenwolf (1993), Manasek (1968), Patten (1957), Romanoff (1960) and Viragh et al. (1989), the human from Hamilton and Mossman (1972) and Sissman (1970), the frog from Sater and Jacobson (1990) and the zebrafish from Stainier and Fishman (1992).

Zebrafish heart development proceeds through very similar steps as in amniotes using well-conserved genetic pathways, in a much shorter time frame.

Conserved Cardiovascular Developmental Events



Heart development and regeneration. 2010.

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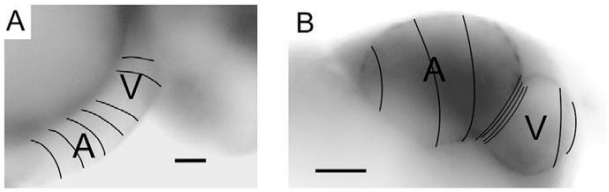
Zebrafish Cardiac Conduction system

Even with only a two-chambered heart, a functional CCS is necessary to initiate, maintain and coordinate heart rhythm so that synchronized contraction can take place.



A ring of AV conduction tissue at 40hpf (Milan et al., 2006)

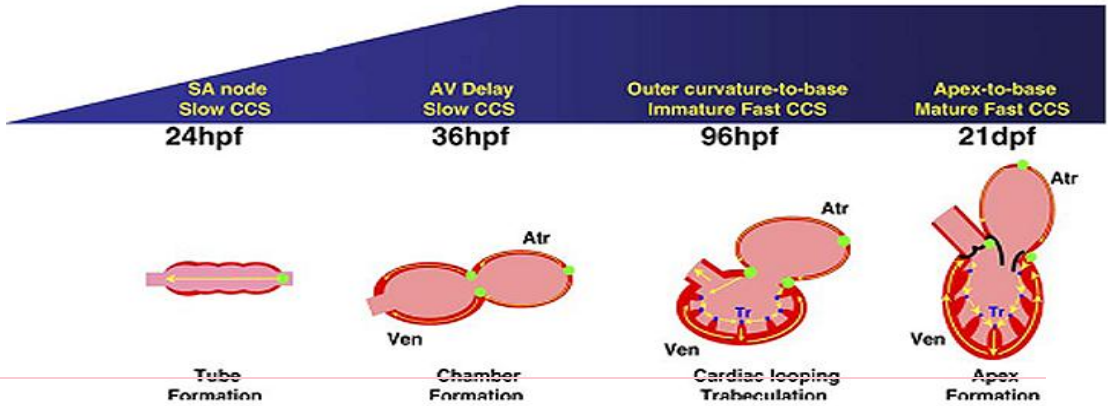


Fig 1.3. The four stages of CCS development in zebrafish (Neil et al., 2008).

Stage 1 (20–24 hpf), a linear activation wave travels across the heart tube from the sinus venosus to the OFT

Stage 2 (36–48 hpf), a significant AV conduction delay develops;

Stage 3 (72–96 hpf), an immature fast conduction network develops within the ventricle;

Stage 4 (21 dpf), this fast conduction network fully matures resulting in appearance of an apex-to-base activation pattern.

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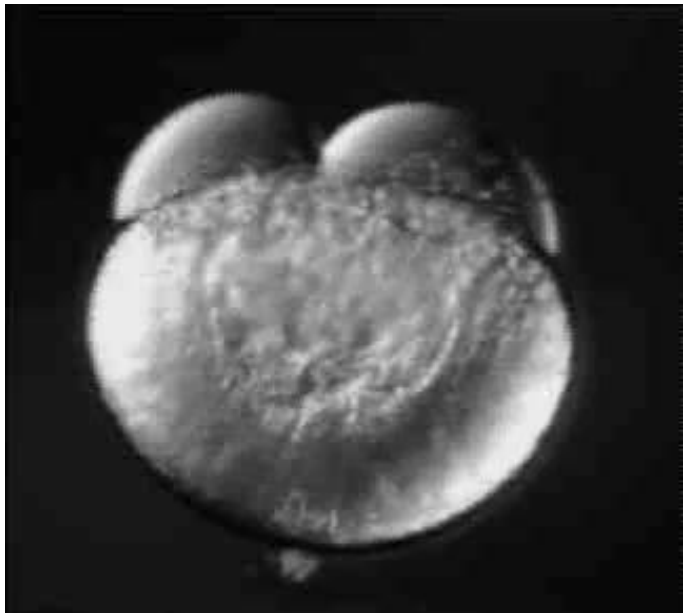
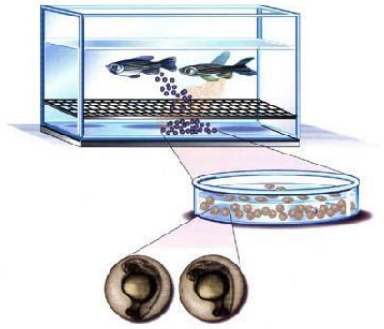
Advantages of Zebrafish as a Model Organism

- Vertebrate Model
- High fecundity (200-300 eggs per female per week)
- Short generation time of 3-4 months
- External development
- Optical clear embryos ideal for *in vivo* imaging
- Rapidly growing embryos
- Amenable to genetics and embryological manipulation
- Diurnal sleep cycle
- Infrastructure available :
  - Sequenced genome
  - Characterized mutants
  - Transgenic lines
- Conserved genetic pathway and function
- Comparable physiology, pharmacological response to drugs



(Karlstrom and Kane, 1996)



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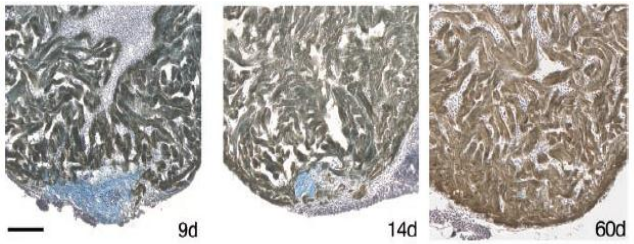
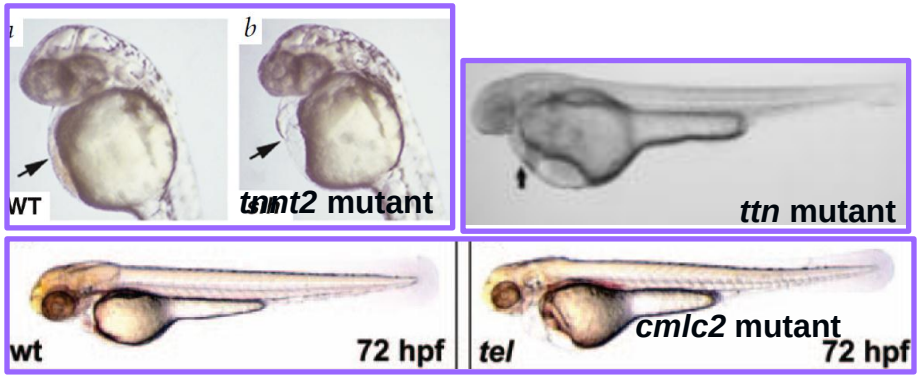
Advantages of Zebrafish as a Model Organism for Heart Development and Disease

- Ability to survive without circulation for days
- Ability to regenerate a damaged heart
- Easy non-invasive identification of cardiac abnormalities, pericardial edema, heart rate, heart rhythm etc.
- Techniques and technologies developed to assess cardiac function e.g. optical mapping, electrocardiogram, video microscopy etc



ENU Mutants defective in heart function



(Sehnert et al., 2002; Xu et al., 2002; Rottbauer et al., 2006)

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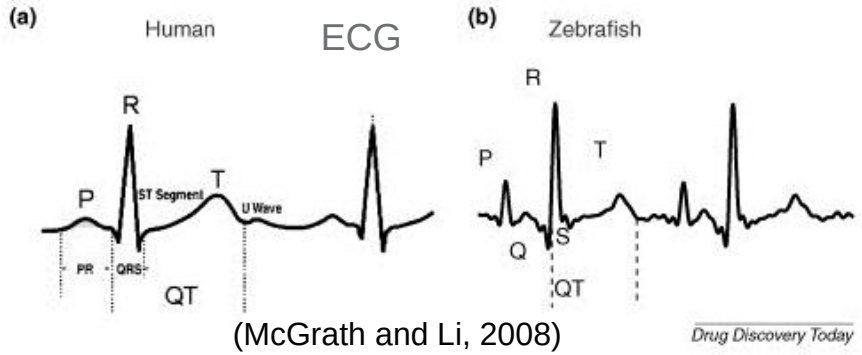
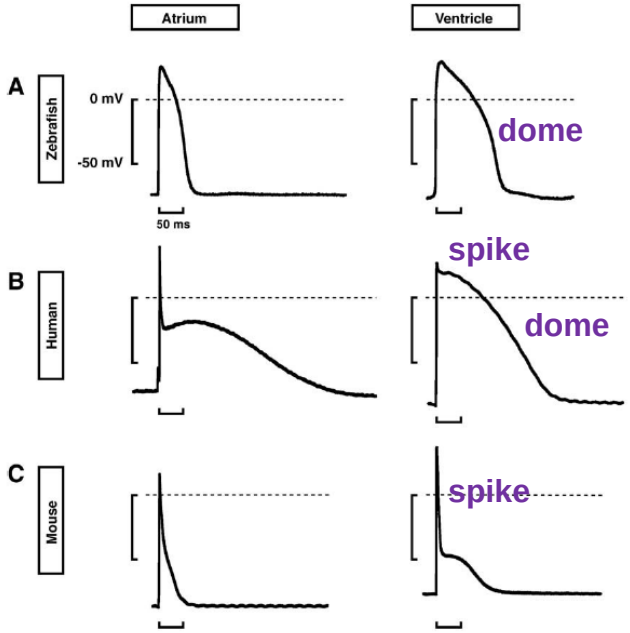
Advantages of Zebrafish as a Model Organism for Heart Development and Disease



(McGrath and Li, 2008)

	HR (bpm)	QTc (msec)
Human	60-100	300–450
Zebrafish	80-200	300-440
Mouse	300-600	83-96

Simplified yet conserved cardiac conduction system



Action potential

(Nemtsas et al., 2009)

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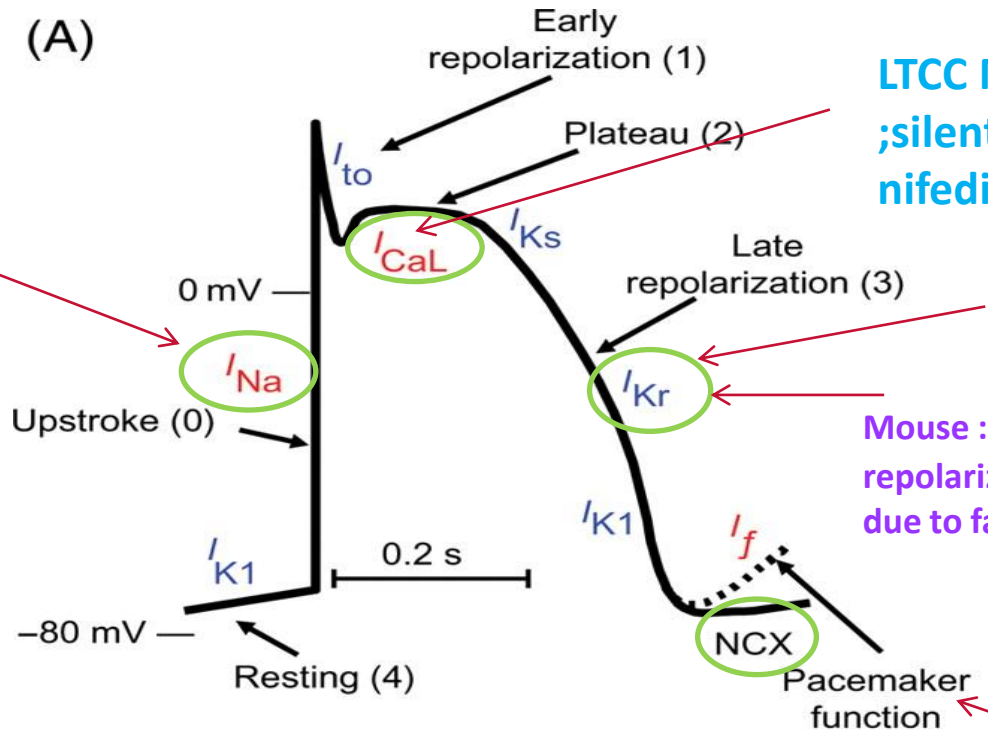
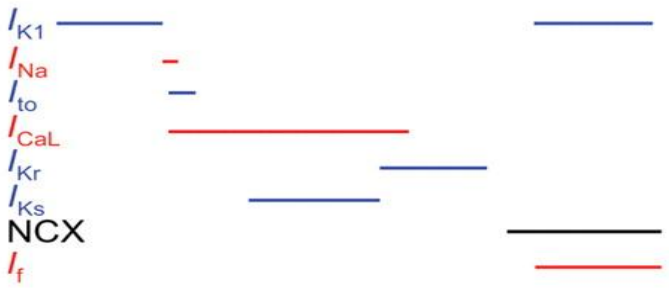
Action potential of zebrafish ventricular cardiomyocytes





MO
TTX

LTCC Mut (atrial cells beat w/o coordination ;silent ventricle)
nifedipine

Mut (2 ERG mutants – long ; short QT)
E4031

Mouse : *I*to, major repolarization current due to fast HR

NCX mut (atrial fibrillation, arrhythmia; silent ventricle)

Fish mutants available

(Nemtsas et al., 2009)

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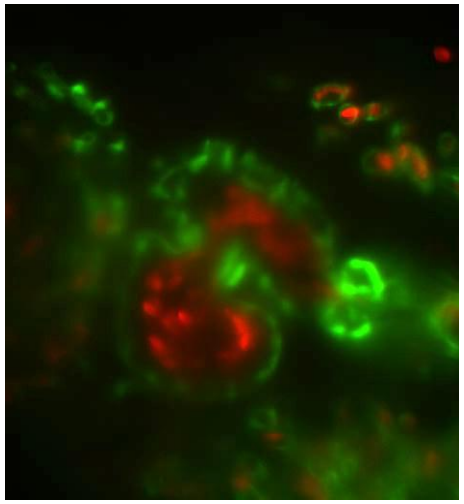
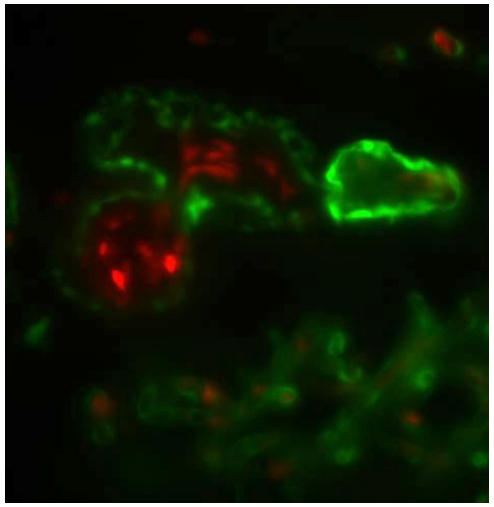
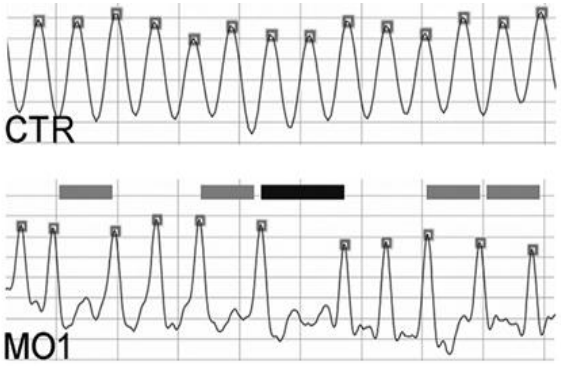
Assessing Cardiac Arrythmias

CTL



popdc2 morphant





popdc2 morphants display cardiac conduction abnormalities

Kirchmaier et al. (2012)

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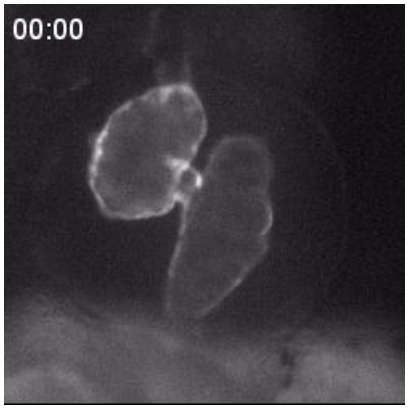
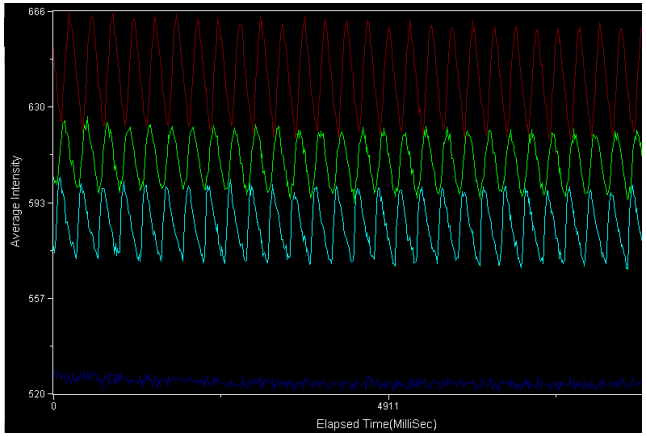
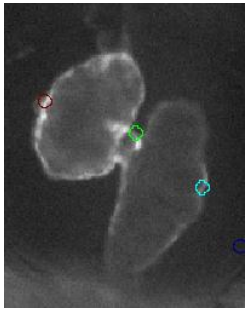
Assessing Cardiac Arrythmias

Calcium Imaging Of Zebrafish Embryonic Heart

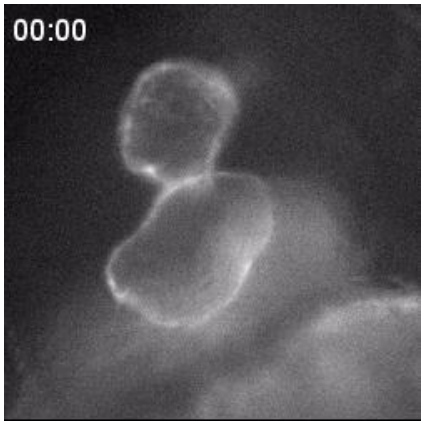
Control

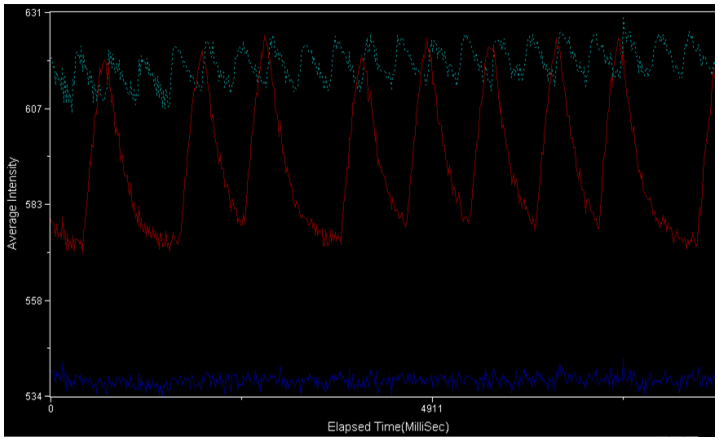


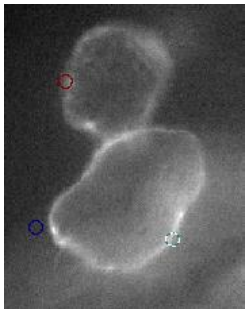




morphant



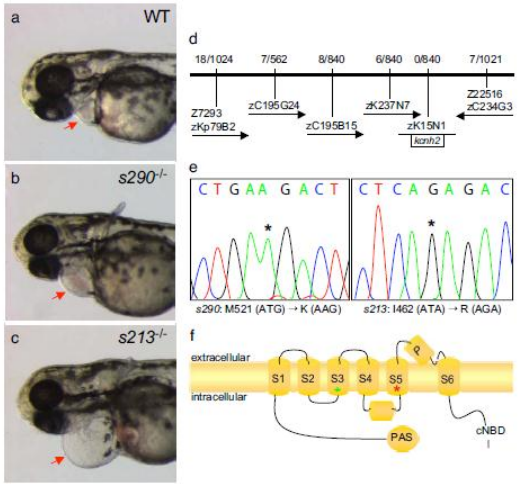


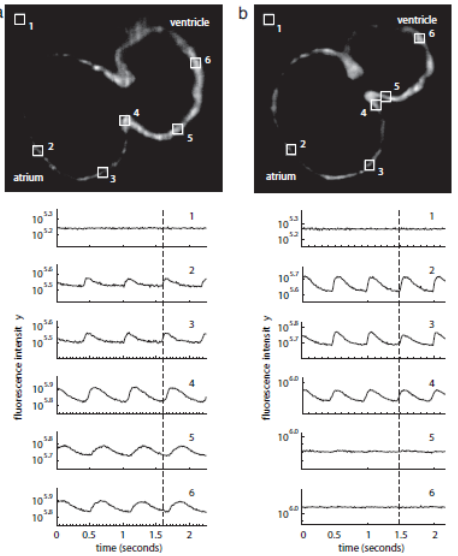


Zebrafish model for human long QT syndrome

Rima Arnaout[†‡], Tania Ferrer[§], Jan Huisken[†], Kenneth Spitzer[§], Didier Y. R. Stainier[†¶], Martin Tristani-Firouzi[§¶], and Neil C. Chi[†¶]

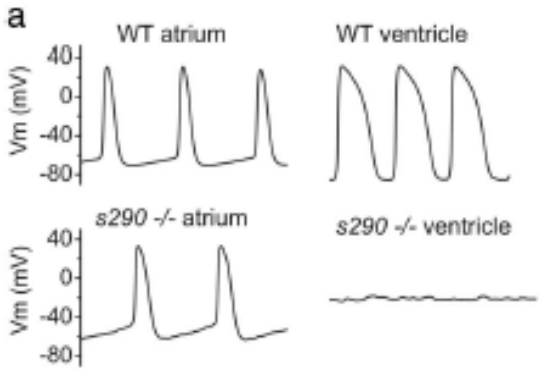
Silent ventricle mutant deficient in *kcnh2*



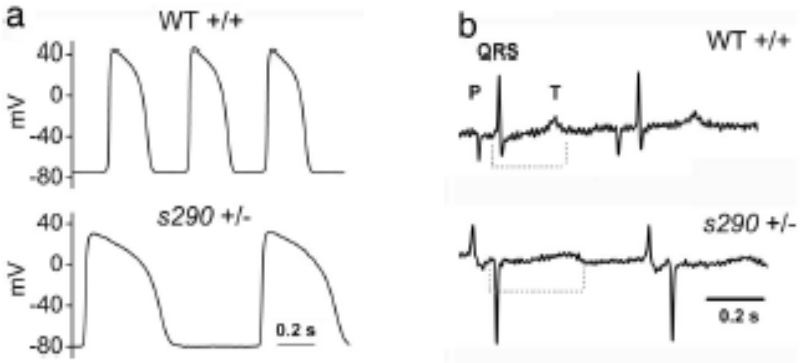


Calcium waves measurement using calcium reporter transgenics

kcnh2$^{-/-}$ ventricle does not generate Aps.



kcnh2$^{-/+}$ manifest delayed ventricular repolarization



APs ECG

(PNAS,2007)

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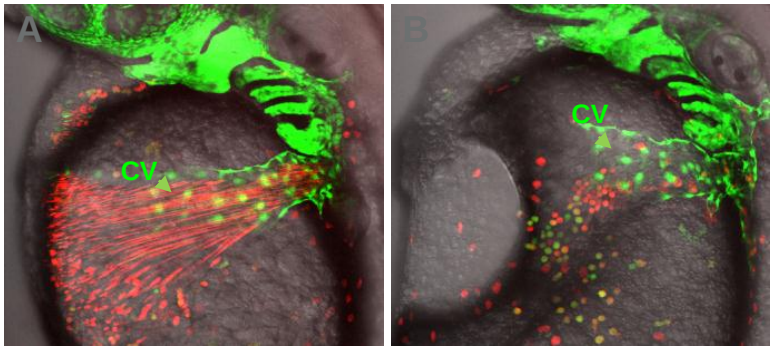
Assessment of Cardiac Function

Blood Flow

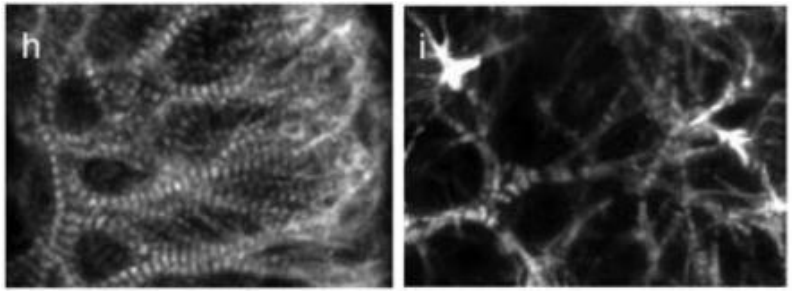
CTL Morphant



Tg(fli1a:EGFP) - blood vessel
Tg(gata1:dsRed) - blood cells

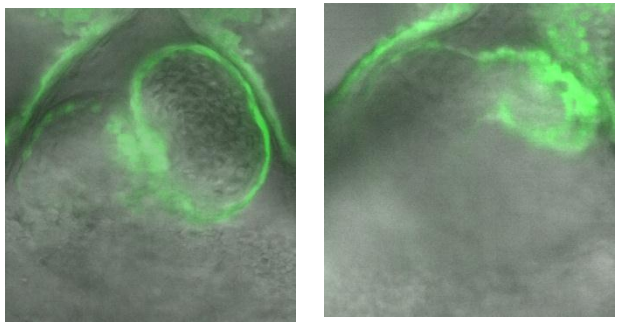
Sarcomeric structures

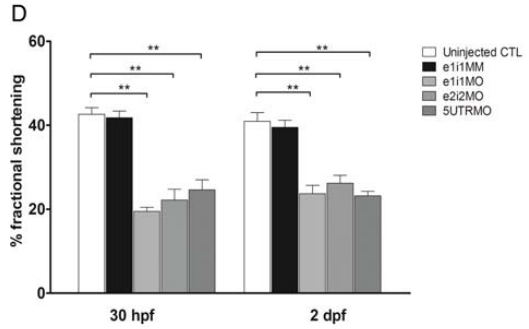
CTL Morphant



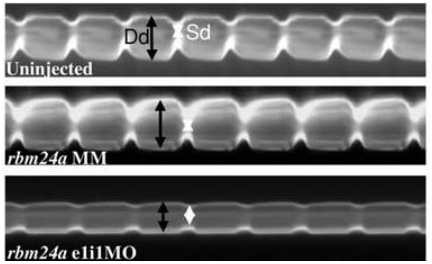
Fractional shortening – a measure of cardiac contractility

CTL rbmMO





M mode



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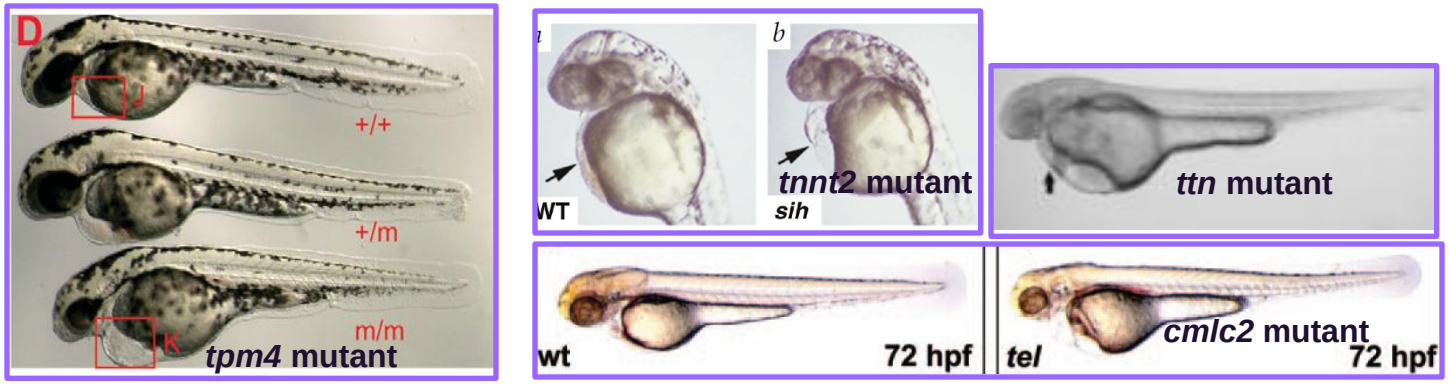
Zebrafish as Model for Cardiomyopathy

- Dilated cardiomyopathy (DCM), familial hypertrophic cardiomyopathy (HCM)
- Hallmarks of Cardiomyopathy:
  - Dilated/hypertrophy of the ventricle(s)
  - Diminished myocardial contractility
  - Myocardial disarray
- Mutations in genes encoding proteins of the sarcomere assembly apparatus: *MYH7, MYL3, MYL2, TNNT2, TNNI3, TPM1, TNNC1, TTN and MYBPC3*
- Zebrafish mutants as model of cardiomyopathy
  - Contractility defect, circulation defect, pericardial edema
- Limitations: Most human cardiomyopathy occurs in adults and involves cardiac remodeling
  Adult models of cardiomyopathy



(Sehnert et al., 2002; Xu et al., 2002; Rottbauer et al., 2006; Zhao et al., 2008)

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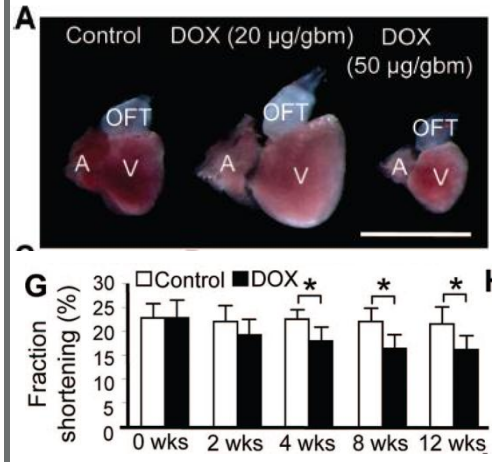
Adult models of cardiomyopathy

Haploinsufficiency of Target of Rapamycin Attenuates Cardiomyopathies in Adult Zebrafish

Yonghe Ding, Xiaojing Sun, Wei Huang, Tiffany Hoage, Margaret Redfield, Sudhir Kushwaha, Sridhar Sivasubbu, Xueying Lin, Stephen Ekker and Xiaolei Xu

Circ Res. 2011;109:658-669; originally published online July 14, 2011;
doi: 10.1161/CIRCRESAHA.111.248260

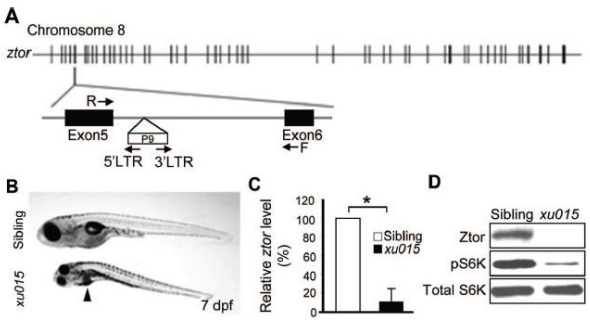
To evaluate cardioprotective function of TOR signaling inhibition



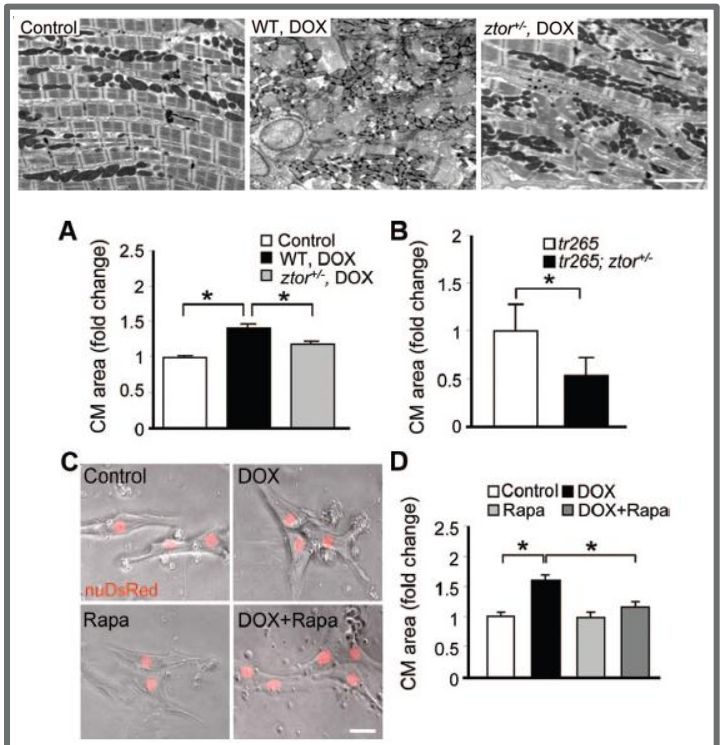
Doxorubicin inducible cardiomyopathy model

DOX Induces Progressive and Pathological Cardiomyopathy in Adult Zebrafish

A cardioprotective function of TOR signaling inhibition has been suggested by pharmacological studies using rapamycin, a specific inhibitor of TOR but not demonstrated by genetic analysis.



zebrafish target of rapamycin mutant (*ztor*) generated



TOR haploinsufficiency attenuates DOX-induced cardiomyopathy and cardiomyocyte hypertrophy.

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The regenerative capacity of Zebrafish

- Extraordinary capacity to regenerate throughout adult life
- Regenerate brain, fins, scales, retina, spinal cord and kidney among other internal organs
- humans myocardial infarction, necrotic myocardium is replaced by non-contractile scar tissue and fail to properly repair or regenerate damaged cardiac tissues
- In contrast, zebrafish hearts regenerate after substantial injury or tissue damage.

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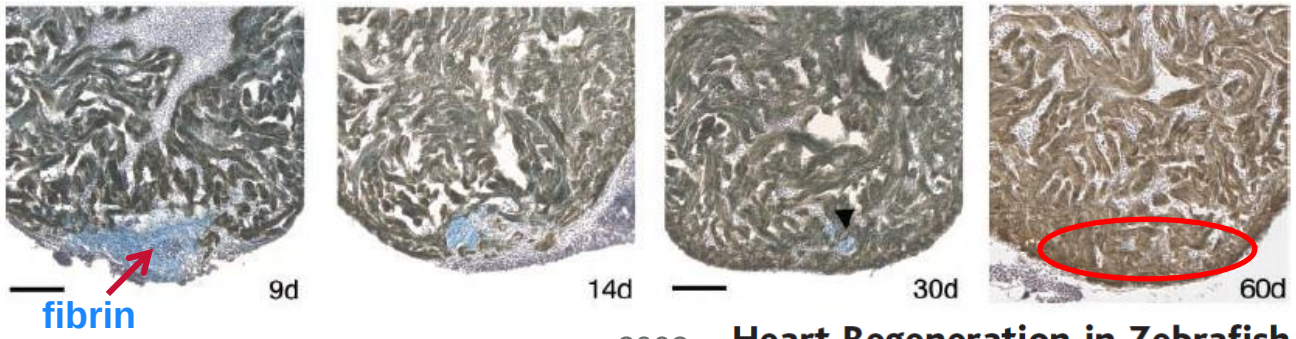
Zebrafish Heart Regeneration



2002 **Heart Regeneration in Zebrafish**

Kenneth D. Poss,* Lindsay G. Wilson, Mark T. Keating*

Zebrafish regenerate its heart after 20% ventricle amputation by 2 months

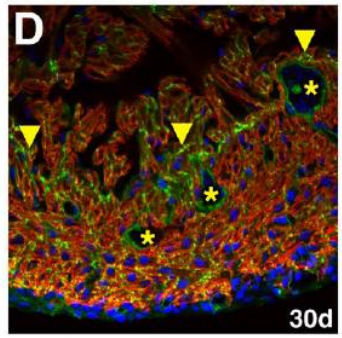
Origin of new cardiomyocytes???

1. Proliferation from fully differentiated cardiomyocytes
2. Proliferation from undifferentiated progenitors
3. Dedifferentiation followed by proliferation of cardiomyocytes

Other cell types involved? epicardium? endocardium?

Signalling pathway?

Functional recovery?



Blood vessels

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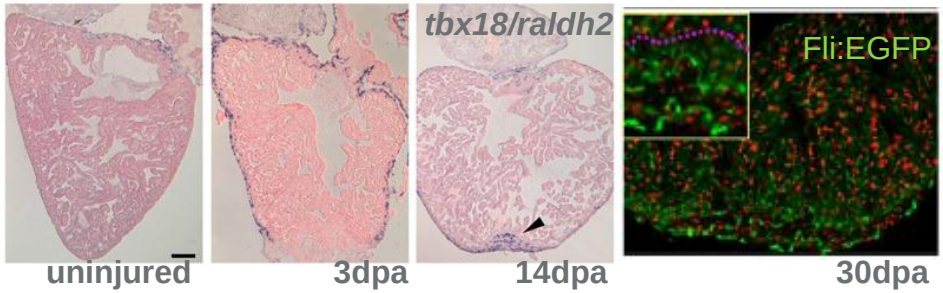
Zebrafish Heart Regeneration

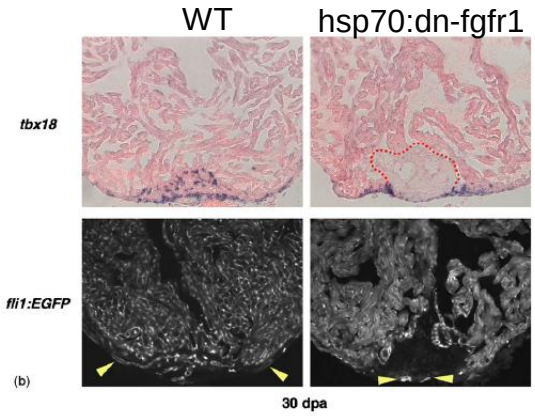
Role of epicardium and signaling pathway involved

Organ-wide activation of epicardial cells, invasion, EMT, vascularization



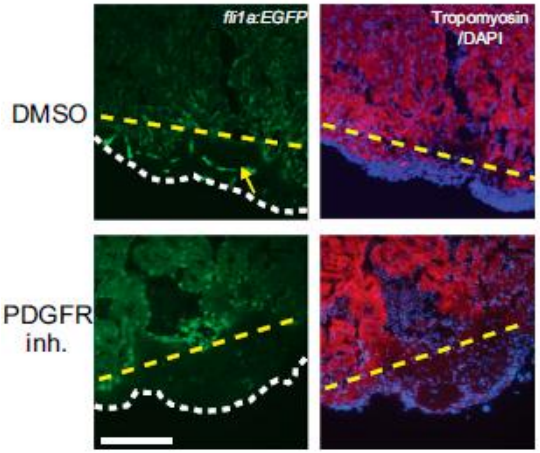
Activation of the epicardium and neovascularization of the regenerating myocardium requires FGF and PDGF signalling.

Fgfr inhibition blocks epicardial EMT, disrupting coronary neovascularization and arresting regeneration



PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts 2010

Jieun Kim[a,1], Qiong Wu[a,1,2], Yolanda Zhang[a,1], Katie M. Wiens[a,1], Ying Huang[a], Nicole Rubin[a], Hiroyuki Shimada[b], Robert I. Handin[c], Michael Y. Chao[d], Tai-Lan Tuan[a], Vaughn A. Starnes[a], and Ching-Ling Lien[a,3]



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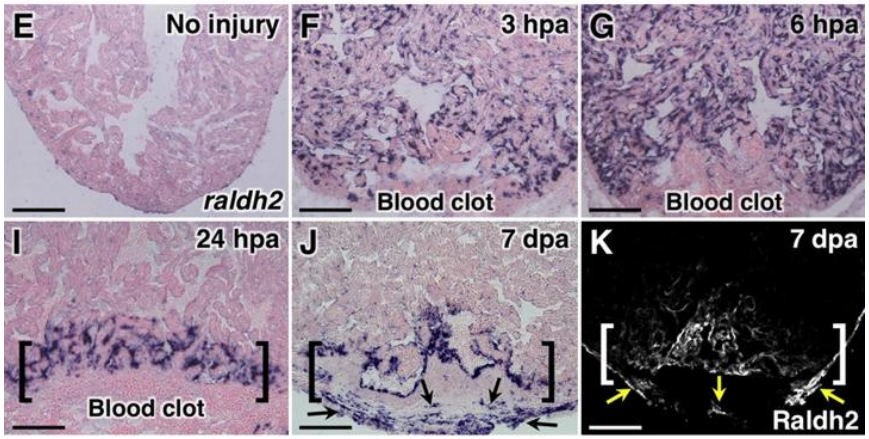
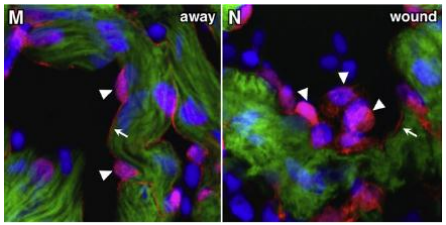
Zebrafish Heart Regeneration

Role of endocardium and signaling pathway involved

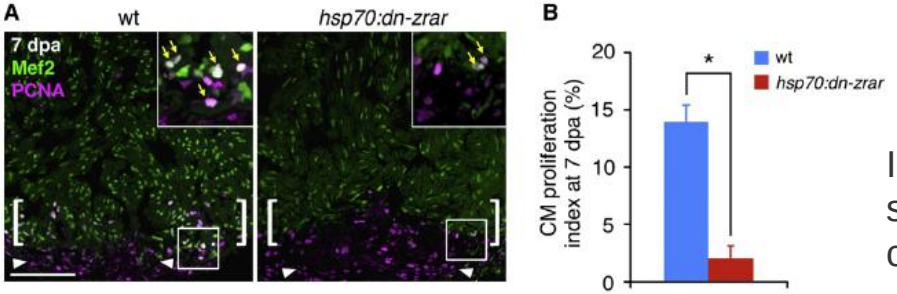


Within hours of ventricular resection, *raldh2,* which is a key enzyme involved in the synthesis of retinoic acid (RA) and promotes cardiac myocyte proliferation,is upregulated.



endocardium undergoes extensive changes in morphology

Endocardial activation is required for cardiomyocyte proliferation



Induced transgenic inhibition of RA signalling blocked regenerative cardiomyocyte proliferation.

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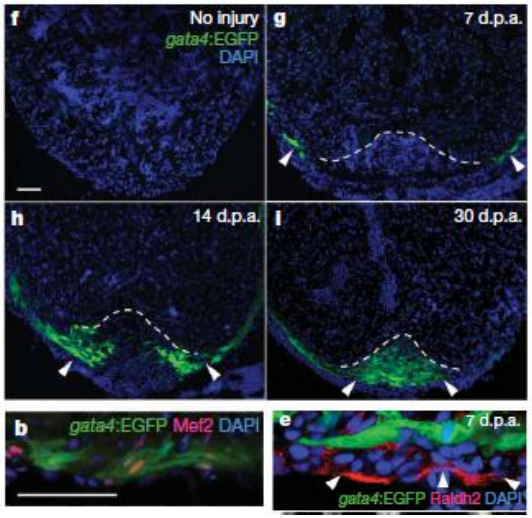
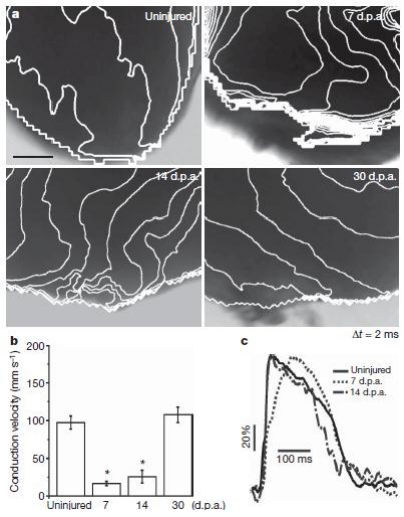
Zebrafish Heart Regeneration

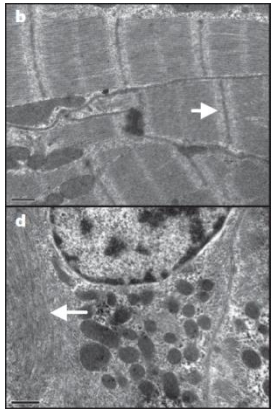
Source of new cardiomyocytes



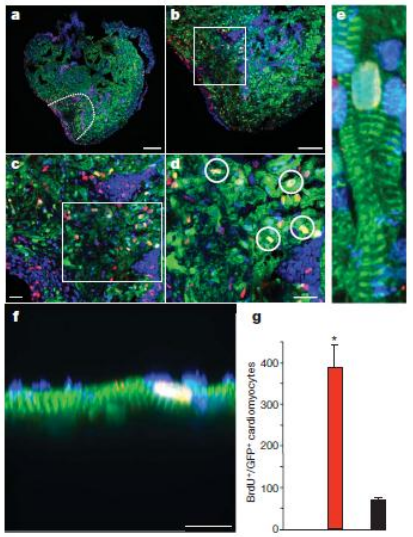
Cardiomyocytes (not epicardium) marked by gata4:EGFP are activated by injury and proliferate at the injury site.



Electrical conduction is re-established.



Pre-existing cardiac myocytes undergo dedifferentiation and is accompanied by sarcomeric disassembly.



Dedifferentiated cardiac myocytes re-enter cell cycle and proliferate to supply new functional cardiac myocytes.

(Kikuchi et al., 2010)

(Jopling et al., 2010)

The source is not cardiac progenitors, epicardium, endocardium but pre-existing cardiac myocytes that dedifferentiate to undergo proliferation.

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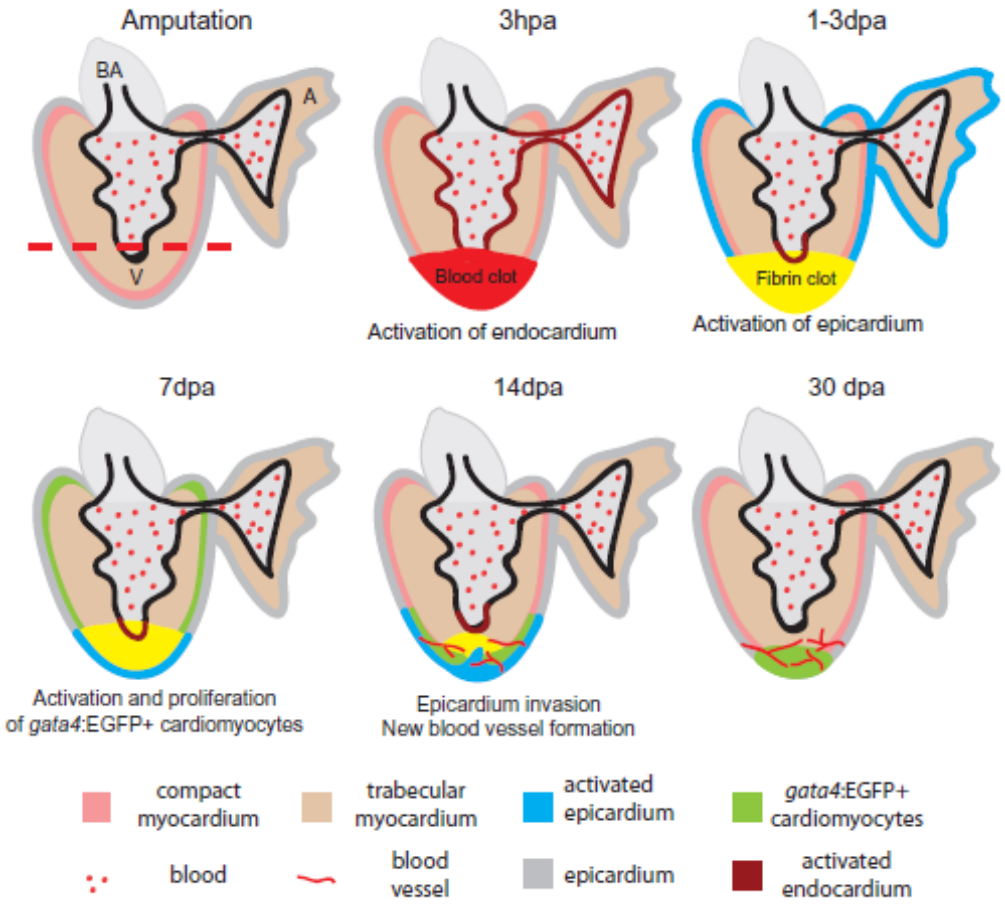
Summary Of Events During Zebrafish Heart Regeneration.

- Right after amputation, a blood clot forms.
- Within hours, the endocardium is activated and shows morphological and gene expression changes.
- At 1–3 dpa, the blood clot becomes a fibrin clot. The activated *raldh2* expression in endocardium becomes localized to the injury site.
- At the same time, the epicardium is activated and expresses embryonic markers.
- At 7 dpa, the epicardium encloses the apex and starts to invade the fibrin clot, while a population of *gata4*:EGFP positive cardiomyocytes appears at the sub-epicardium and begins to proliferate.
- At 14 dpa, the *gata4*:EGFP-positive cardiomyocytes localize to the apex and newly formed blood vessels vascularize the newly formed myocardium.
- By 30 dpa, the myocardium is almost fully regenerated.
- The new blood vessels vascularize the new myocardium.



(Lien et al., 2012)

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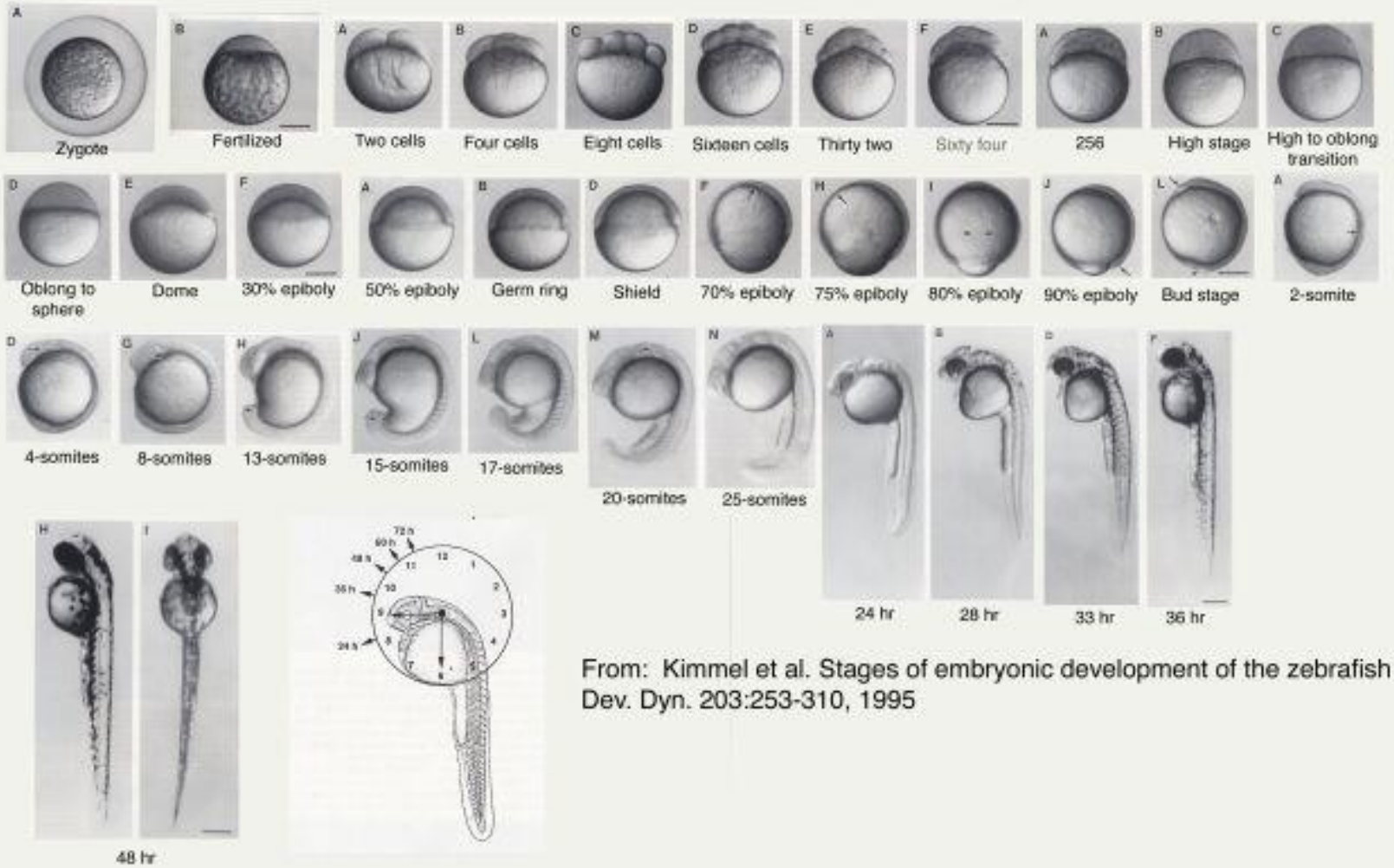
Zebrafish Heart Regeneration

new cardiac injury models

Methods of inducing cardiac Injuries	% Myocardium damaged	Recovery time	Scarring	Apoptosis	Advantages
Ventricular Resection	20%	2 months	Minimum with low collagen content	Some apoptosis and necrosis along the amputation plane	Well established method
Cryocautherization	25%	>3 months	Yes	Apoptosis and necrosis throughout the heart	Mimics human myocardial infarction
Genetic ablation by diphtheria toxin A chain (DTA)-induced cytoxicity	60%	< 2 months	No	Apoptosis throughout the ventricle uniformly at 3 and 5 dpi	robust cellular and molecular responses by endocardial, immune, epicardial and vascular cells; 2 to 4 fold higher cardiomyocyte proliferation; eliminates the need for doing microsurgery,



From: Kimmel et al. Stages of embryonic development of the zebrafish
Dev. Dyn. 203:253-310, 1995