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Animal models of cystic fibrosis in the era of highly effective modulator therapies

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Abstract

Few human genetic diseases can rely on the availability of as many and as diverse animal models as cystic fibrosis (CF), a multiorgan syndrome caused by functional absence of Cystic Fibrosis Transmembrane Regulator (CFTR). The recent development of highly effective CFTR modulator drug therapies simultaneously highlighted the remarkable clinical improvement achievable with these treatments, the lack of therapeutic alternatives for non-responders, and the need to understand the kinetics of disease upon early life/chronic treatment. These advances have rekindled efforts to leverage animal models to address critical knowledge gaps in CF. This paper provides a concise overview of the areas of interests for therapeutic intervention in the current CF landscape, focusing on the contributions of *in vivo* models to understand CF pathogenesis, identify therapeutic windows, and develop novel therapies for all CFTR mutations.

Keywords

Cystic Fibrosis; animal models

Introduction

Cystic Fibrosis (CF) is a monogenetic disease caused by absence of functional Cystic Fibrosis Transmembrane Regulator (CFTR), an anion channel that directly regulates chloride and bicarbonate secretion/absorption across epithelia in many organs. Defects in CFTR function may arise from aberrant transcription/translation, folding/degradation, trafficking/stability in the plasma membrane, or gating/conductance. Main manifestations of the disease involve: 1) airway mucus obstruction and inflammation with predisposition to bacterial infections and recurrent exacerbations, leading to progressive tissue damage and respiratory failure; and 2) gastrointestinal, pancreatic and hepatic dysfunctions, which lead to malabsorption and affect intestinal transit, growth, and overall metabolism(1).

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CFTR orthologs are expressed in vertebrates, from cartilaginous fish onward (2, 3). This has allowed the development of CF animal models in several species, which have been used to investigate CF pathophysiology and test therapeutic treatments. In order of first appearance in the published literature, CF researchers have generated animal models in the following species: mouse(4, 5), pig(6–10), ferret(11, 12), rat(13–17), zebrafish(18, 19), sheep(20, 21), drosophila(22), and rabbit(23, 24). Other non CFTR-directed animal models have been instrumental to study particular aspects of CF pathophysiology, *e.g.*, defective airway mucus clearance (*Scnn1b*-Tg mice(25–27), elastase-treated sheep(28–31) or mice(32, 33)) or to simplify complex system biology, *e.g.*, mucosal surface immunology (*Xenopous laevis* tad pole(34, 35)). Far from being redundant, this variety reflects an effort to provide the CF research community with multiple tools to study CF pathophysiology or therapeutic options, and to overcome the shortcomings inevitably associated with any individual animal model in terms of organ-specific phenotypes, cost, and tractability, as extensively reviewed in the recent past(36–40).

The advent of highly effective modulator drug therapies (HEMT), small molecules that aid mutant CFRT intracellular trafficking and support its function once it reaches the plasma membrane, has transformed health outcomes and quality of life for a large fraction (~90%) of people with CF (PwCF)(41–43). These highly beneficial therapies are projected to further improve longevity of PwCF and their stark success has poised the scientific community to address the lack of treatment for the 10% of PwCF who do not respond to HEMT and to explore the long-term implications and new challenges arising from a longer life with CF. Notably, none of the currently available HEMT directly stemmed from animal model research. So, how relevant are CF animal models in the HEMT era and can they be harnessed to address emerging CF research priorities? The ongoing PROMISE study(44), a large multidisciplinary study focused on the impact of triple combination therapy (elexacaftor/tezacaftor/ivacaftor) on the US population age 6 and older, highlighted specific “areas of interest” as emerging research priority in CF (*i.e.*, clinical change, mucus biology, microbiology, inflammation and host response, gastroenterology, endocrinology, liver disease, nasal airway epithelial cell function, and pediatric inclusion). These priorities are also reflected in the Cystic Fibrosis Foundation drug development pipeline (https://apps.cff.org/trials/pipeline) where the primary therapeutic outcomes involve: restore CFTR function; enhance mucociliary clearance; develop novel anti-inflammatory and anti-infective strategies; and support nutritional/gastrointestinal health. Arguably, animal models provide the unique opportunity to study how CFTR dysfunction affects multiple organ systems, how disease phenotypes relate to each other, and how they are influenced by non-CFTR-related modifiers, *e.g.*, genetic background(45), microbiota(46), sex(47), age(48), and environmental exposures(49). The purpose of this brief review is to summarize the ongoing contribution of CF animal models’ research to the aforementioned priorities, with reference to recently published papers.

Restoration of CFTR function

Pharmacologic correction of defective CFTR function is achievable provided that 1) the mutant CFTR protein is expressed in the target organ, and 2) the mutant CFTR is responsive to the modulation. About 7% of PwCF carry severe mutations (nonsense/missense, splice-

site, frameshifts, insertions/deletions) that lead to complete absence of CFTR protein, and are thus non-responsive to HEMT. The challenging goal of introducing functional CFTR in affected tissues using gene therapy approaches has been heavily pursued using CF animal models(50, 51). Wild-type animals have been used to evaluate safety and efficiency of different vectors or delivery methods (e.g., adeno-associated vector in ferret(52), lentiviral vectors in marmoset(53), microspray administration in neonatal pigs(54)). Mutant CFTR-based models have been used to evaluate restoration of CFTR activity *in vivo*. Functional readout varied depending on the model used and include normalization of pulmonary function parameters in $CFTR^{tm1Unc}$ KO mouse treated with chemically modified hCFTR mRNA(55), increase in basal nasal potential difference towards WT levels in 510X CF rat treated with a tagged CFTR lentiviral vector(56), and restoration of transepithelial Cl^- current in freshly excised airways from gut-corrected CF pig treated with an integrating adenovirus-based vector expressing hCFTR (57). Models recapitulating key features of CF lung disease, *i.e.*, airway mucus obstruction and inflammation, have been used to study barriers to vectors' delivery(58) and stability(59) in inflamed and obstructed airways. Proof-of-concept studies in mice(60) also supported the feasibility of *in vivo* cell-based therapy, where genetic correction is achieved *ex vivo* in suitable regenerative cells which are then transplanted to the affected organ(61).

Due to their relatively low maintenance cost, fast life cycle, and amenability to complex gene editing manipulation as compared to larger models (*e.g.*, pigs, ferret, sheep, rabbits), rodents have been the species of choice to generate screening tools for read-through pharmacologic therapy against nonsense/missense mutations. The G542X stop mutation has been introduced in mouse(62) and rat(15). This mutation has also been introduced in sheep with the goal of using this neonatal lethal model to test *in utero* therapies (21). In an effort to provide screening platforms with the closest DNA and protein sequence similarity to hCFTR, "humanized" CF mice have been generated by transgenic expression of hCFTR on a mCFTR KO background (63). In this model, multiple copies of hCFTR were integrated in a single insertion site, making further mutant models based on this strain suitable to test pharmacologic interventions (e.g., hCFTR G551D, F508del, G542X, W1282X, 3849+10kb C>T), but complicating the application of gene editing approaches. This limitation has been recently overcome by using exon replacement strategies that allow for tailored substitution of the endogenous mouse sequence with the human one, obtaining a hybrid CFTR (exon 11: WT, F508del; exon 12: WT, G542X, and R553X, Hodges, C. *et al,* Abstract 662 North American Cystic Fibrosis Conference, Virtual event, November 2021, *Journal of Cystic Fibrosis* Vol. 20, Supplement S314).

Among CF animal models, only ferret(11), pig(6), sheep(21), and rabbit(23, 64) CFTR is naturally responsive to modulators of hCFTR, likely due to a higher degree of sequence and structural homology as compared to other species. Pharmacologic rescue of CFTR in animal models can be used to query critical issues that would be intractable in human subjects, such as effective therapeutic windows, spatio-temporal patterns of disease development, and effect of treatment withdrawal. A remarkable example for these studies featured in-utero and postnatal treatment of ferrets carrying the G551D mutation with the potentiator ivacaftor (VX-770)(12).Resulting data support the efficacy of early intervention to prevent perinatal manifestation of the disease (pancreas, intestine, growth), and highlights how

therapeutic regimens need to be maintained to prevent reoccurrence of disease (lung, pancreas). An important corollary of this study is that using the outbred ferret model carrying a defined genetic mutation (G551D) it was possible to appreciate the contribution of disease modifier genes in the manifestation of disease and response to therapy (G551D homozygous ferret with pancreatic insufficiency regardless of VX770 treatment). G551D humanized rats have also been used to test the effect of ivacaftor on age-dependent airway mucus abnormalities(65) and inflammation(66). Notably, accumulating evidence suggest that CFTR function is required for proper organ development, including cartilaginous airways of CF pigs (67), rats (13), and mice (68), or nasal sinuses in CF pigs(69). Congenital absence of vas deferens has been documented in CF ferrets (12), rats(70), rabbits(23), and sheep(21), but not in CF mice, whereas CF pigs exhibit high penetrance of vas deferens and epididymal atresia(71), suggesting species- and organ-specific requirements. These results, along with the increasing availability of different animal models susceptible to pharmacological correction of CFTR, suggest that a range of CFTR function-dependent phenotypes can be exploited to study the developmental components of CF disease.

Mucus biology and mucociliary clearance

Luminal obstruction with inspissated mucous secretions characterize the pathologic presentation of CF in several organs (upper and lower respiratory tract, gastrointestinal tract, pancreas, liver, gallbladder, female and male reproductive tract) and has been directly linked to absence of functional CFTR-mediated Cl^- and $HCO3^-$ secretion. Understanding of the specific mechanisms and tissue/molecular components involved in the generation and failed clearance of these thick secretions is an active area of investigation (72–75). Abnormalities in airway mucus have been detected in bronchoalveolar lavage from young children with CF, were associated with inflammation, and preceded bacterial infection (76), suggesting a causal role in the pathogenesis of obstructive CF lung disease. This central role has been substantiated by studies in CF animal models, including pigs(7, 77), ferret(78), and mouse(27). The general components of the mucociliary clearance system (mucus producing, secretory, and ciliated cells) are present in all CF animal models, although relevant anatomic and cell composition differences must be considered in comparative studies. For examples, airway submucosal glands are associated with cartilaginous airways throughout human airways, with a similar distribution observed in pigs and ferrets. In contrast, submucosal glands are localized in the trachea in rats, they are further restricted to the most proximal portion of the trachea in mice, and are completely absent in rabbits(79). These differences have been exploited to investigate the contribution of submucosal gland vs. superficial epithelia in the pathogenesis and progression of CF lung disease, and evidences so far suggest that the presence of submucosal glands is critical to develop spontaneous and chronic obstructive lung disease (pig and ferret), whereas failure to clear secretions from the smaller conducting airways might be responsible for creating early, heterogeneously distributed “pathologic niches” in the deeper lung(23, 80). Of note, scRNAseq technologies have recently been used to define the cellular and molecular landscape of surface epithelium and submucosal glands in CF vs. WT pigs at birth(10, 81), establishing a critical, initial timepoint for a much-anticipated time course analysis.

In larger CF animal models, lung disease progression and efficacy of therapeutic interventions can be studied longitudinally using imaging modalities similar to humans, like computer tomography (*e.g.*, ferret (78, 82), pig(77)), whereas other techniques had to be devised to study mucociliary clearance in species with smaller lung volumes. Given the many variables that affect mucociliary clearance *in vivo*, recent efforts have been focused on developing measurements that can be made on intact tissue or *in situ* to avoid perturbation of the environment. In particular, intact tracheas have been studied with microoptical coherence tomography (μOCT), which allows measurement of thickness of secretions, ciliary beat frequency, mucociliary transport, and submucosal inflammation (14, 83, 84), or fluorescence particle tracking (85). Although sophisticated methods to quantitatively assess obstructive lung disease in the lower airways of smaller species are under development (86–88), the vast majority of studies relies on histological and morphometric assessment of airway mucus burden and inflammation. Specifically, morphometric analyses have been used to quantify airway mucus burden in *Scnn1b*-Tg mice treated with mucus-mobilizing agents(89–91), elastase-treated WT mice(32), or CF rats challenged with *P.aeruginosa* embedded in agar beads (Birket, S. et al. Abstract 72 North American Cystic Fibrosis Conference, Denver, October 2018, *Pediatric Pulmonology* Vol. 53, Supplement 2).

Infection

Although defective airway mucus clearance likely plays a major role in initiating CF lung disease, chronic bacterial airway infections are the leading cause of morbidity and mortality in PwCF. Spontaneous, chronic airway bacterial infections with a range of pathogens including *Staphylococcus*, *Streptococcus*, and *Enterococcus* have been reported in CF ferrets(11, 92) and CF pigs(7, 93). Overall, rodent models do not exhibit chronic airway bacterial infection, with the only two notable exceptions being 1) congenic C57BL/6 *Cftr*^{tm1kth} mice, in which a proportion of mice (1/3) exhibit colonization by *Bordetella pseudohinzii associated with decreased respiratory rates* (94), and 2) *Scnn1b*-Tg mice which are susceptible to spontaneous colonization by oropharyngeal microflora in the early post-natal period (5–10 post-natal days), but become resistant as they age (27). These models allow study of factors facilitating the establishment and the evolution of airway bacterial infection from the perspective of both the host and the pathogens, as well as pharmacological interventions. Several models of experimentally-induced bacterial infection have also been developed to test specific contributions of host and pathogen factors, with an important distinction between “chronic” vs. “acute” protocols(95). In the absence of a CF-like muco-obstructed and likely hypoxic milieu, *i.e.*, in WT animals, chronic (*i.e.*, lasting more than 1–2 weeks) infections are often obtained by embedding the bacteria in polymer beads which provide a protective microenvironment against mucociliary and immune clearance. Although not without limitations, these models have been critical to demonstrate the contributions of host genetic background(45), to benchmark current therapies (96), and to test novel anti-infective treatments, e.g., phage therapy(97). Acute anti-infectives screening has been performed using aerosolized bacteria models(98), and CF zebrafish embryos(99). Viral infections have been linked to pulmonary exacerbations and progressive decline in lung function, but respiratory viruses routinely cultured during periods of exacerbation in PwCF (*i.e.*, influenza, respiratory syncytial virus, and respiratory virus(100))

have not been systematically studied in CF animal models, likely due to differences in viral tropism across species. Non-tuberculous mycobacteria (NTM) are emerging pathogens for PwCF and are found in ~ 10% of the patient population(101). Pharmacologic screening of anti-NTM compounds or studies investigating the interaction between NTM and the immune system can be performed in non-mammalian models (zebrafish, *X. laevis* tad pole(35)), in mice deficient in innate or adaptive immune mediators (e.g., GM-CFS KO, SCID, and NOD) or transiently immunosuppressed by chronic corticosteroid treatment(102). Integration of these approaches in animal models recapitulating specific features of CF lung pathology could provide further insights for the detection and treatment of old and emerging microbial threats for PwCF.

Inflammation

Chronic inflammation is a hallmark of the multi-organ CF syndrome and it is likely due a combination of recurrent challenges, hyperactive yet inefficient immune responses, and delayed resolution (103). Intrinsic, *i.e.*, due to abnormal CFTR function within the cell, and extrinsic, *i.e.*, due to CFTR-dependent alterations in the cellular milieu, abnormalities have been described for CF neutrophils(104) and macrophages(105). CF pigs and ferrets exhibit spontaneous inflammation in the respiratory and gastrointestinal tracts, and inflammatory markers have been used to track the development and progression of specific organ disease. As these models are amenable to HEMT treatment and might exhibit different levels of organ-specific therapeutic success, it is plausible that they could be harnessed to study the interconnection and developmental dynamics of CF inflammatory responses. In parallel, studies in species considered less exemplary of human CF pathology, like rodents, have highlighted the potential for intrinsic, CFTR-dependent drivers of inflammation. For example, CF mice exhibit an inflammatory imbalance both at baseline (in serum) and after chronic *Pseudomonas aeruginosa* or lipopolysaccharide (LPS) challenge (in the lung) (106–108). Bone marrow transplant experiments have suggested that cell-based supportive therapy with hematopoietic and mesenchymal stem cells could be beneficial to enhance CF immune regulation (109). Of note, a recent report suggests that the muco-obstructed microenvironment associated with CF lung disease promotes epigenetic reprogramming of resident airway macrophages, shifting their transcriptional profile and activity towards a state that is both hypofunctional and hyperinflammatory(110). These findings share some features with recent single cell RNA sequencing (scRNAseq) analyses of CF vs. healthy control sputa that identified dysregulation in pathways associated with phagocytosis and immune cell regulation in CF macrophages and monocytes populations (111). Finally, humanized G551D rats have been used to test whether pharmacologic correction with ivacaftor would restore the hyperinflammatory lung phenotype present in 6 months-old hG551D rats, both at baseline and after LPS challenge, a surrogate for pulmonary exacerbations. A 7-day treatment with Ivacaftor effectively reduced inflammatory markers in bronchoalveolar lavage from naïve hG551D rats, failed to fully revert the effects of LPS stimulation, suggesting that short-term correction is insufficient to regulate the hyperinflammatory response to a stronger stimulus(66).

Gastrointestinal, pancreatic, and liver disease

Although the respiratory pathology is the primary cause of concern for PwCF, dysfunctions in the gastrointestinal (GI) tract, pancreas, and liver significantly increase the disease burden and affect quality of life. Human CF GI disease is characterized by an ~20% incidence of meconium ileus (MI) at birth (112). In adults, gastroesophageal reflex disease (GERD) affects up to 80% of PwCF), distal intestinal obstruction syndrome (DIOS) is commonly diagnosed, and small intestine bacterial overgrowth (SIBO) is found in 30–50% of PwCF (113). Moreover, PwCF suffer from high incidence of esophageal, gastric, liver, gallbladder, and colon cancer, likely linked to chronic inflammation and tissue damage. Absence of CFTR-mediated bicarbonate and fluid transport in pancreatic ducts causes premature acid-activation of the digestive enzymes secreted by acinar cells and impairs flow of these enzymes into the duodenum, causing malabsorption and concomitant pancreatic inflammation and fatty replacement. These pathologic changes begin before birth and affect 85% of PwCF, and the “collateral damage” in the endocrine pancreas leads to CF-related diabetes in 50% of PwCF, who present with worse lung disease, poorer nutritional status, and increased mortality. Similarly, absence of functional CFTR in the cholangiocytes lining the hepatic biliary ducts leads to cholestasis, microgallbladder, inflammation, and progressive liver fibrosis, another leading source of morbidity and mortality. All CF animal models present gastrointestinal abnormalities of different severities (114, 115) likely due to species-specific physiology as well as CFTR and “vicariate” ion channels’ expression patterns. In CF mice, rats, and rabbits the most noticeable GI phenotype is distal intestinal/proximal colon obstruction - analogous to DIOS – which is particularly severe after weaning. These species are typically spared from neonatal MI, pancreatic, and hepatic complications (13, 16, 17, 23, 65, 66, 116), except for congenic C57BL/6J $Cftr^{tm1Unc}$ weaned on a liquid diet and aged to up to 2 years, that present with focal hepato-biliary lesions (48, 117). Administration of osmotic laxatives has been used to improve survival and DIOS symptoms in CF mice and other CF animal models, often in conjunction with targeted dietary modifications (23, 93, 118–120). Genetic “gut-correction”, *i.e.*, gut-targeted, transgenic expression of functional CFTR, has also been used to overcome GI pathology in mice, ferrets, and pigs, although stunted growth (121), pancreas-related (9, 11) or metabolic abnormalities (122) still affect these gut-corrected models. GI disease in CF pigs more substantially mirrors human CF, including MI (100% penetrance, requiring surgery at birth), DIOS, diverticulosis, intestinal atresia/stenosis, gastric ulcerations (prophylactically treated with proton pump-inhibitors), alterations in intestinal muscle motility, severe pancreatic pathology detected at birth that requires life-long pancreatic enzymes replacement therapy (PERT), focal biliary cirrhosis, and micro-gallbladder (6, 8, 123, 124). Similar to CF pigs, CF ferrets have been instrumental in illuminating aspects of CF extra-pulmonary pathophysiology, and windows of opportunity for treatment. In CF ferrets, MI is slightly less penetrant than pigs (75%) and gradually transitions to DIOS; mild exocrine pancreas disease is present at birth but within a month progresses towards severe degeneration which requires PERT and causes bouts of inflammation and remodeling leading to glucose intolerance and CF-related diabetes (125). Studies with G551D ferrets suggest that perinatal DIOS and pancreatic degeneration can be prevented by *in utero* administration of ivacaftor. Conversely, postnatal ivacaftor withdrawal leads to resurgence of these lesions(12). The

recently developed CF sheep(20, 21) also exhibit the full range of human CF-like intestinal, pancreatic, and hepatic complications, and the severity of these lesions likely contribute to the dismal survival of this model (live-born lambs are considered non-viable due to severe MI). Although rabbit express CFTR in the pancreatic ducts and CF rabbit exhibit altered lipid metabolism, histologic analyses revealed only mild focal lesions which appear to be dependent on husbandry and overall health status(23).

Recent attention has been given to the influence of the CF gut microbiota on CF multi-organ pathology and susceptibility to colorectal cancer (126, 127). Bacterial overgrowth has been detected in the small intestine of neonatal and adult CF mice(128, 129) and in CF ferrets (92). Alterations in stool microbiota have been detected in young CF rabbits, although it is not clear if these were due to selective administration of osmotic laxative only to the CF rabbits (130). Of note, germ-free CF mice still develop DIOS, and conversely, the presence of inspissated mucosal secretions actively shapes the gut microbiota composition after experimental re-colonization of these mice(46).

Non-vertebrate CF models are also emerging as tools to study aspects of CF GI pathophysiology. Drosophila lacking the evolutionary conserved miR-263 fail to regulate ENaC function in the gut resulting in dehydration of the intestinal surface, enterocytes activation, and gut bacteria overgrowth (131), a phenotype similar to that of Drosophila lacking the CFTR ortholog CG5789(22). Finally, the kinetics and cell types involved in pancreatic degeneration have been studied in the imaging-friendly CF zebrafish(18).

Conclusions:

The breadth and depth of research efforts using CF animal models clearly indicates that their usefulness is continuously expanding, benefiting from current innovations in analytical and gene-editing technologies. On a concluding note, comparison of scRNAseq data across species suggests that main pathways involved in lung development and repair are conserved across species and can be queried through computational analyses regardless of differences in cell composition and species-specific gene expression(132), providing further confidence in the possibility to translate findings generated in animal models to human diseases.

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Glossary

PwCF	People with CF
HEMT	highly effective modulator drug therapies
μOCT	microoptical coherence tomography
NTM	Non-tuberculous mycobacteria

scRNAseq	single cell RNA sequencing
MI	meconium ileus
GERD	gastroesophageal reflex disease
DIOS	distal intestinal obstruction syndrome
SIBO	small intestine bacterial overgrowth
PERT	pancreatic enzymes replacement therapy

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• of special interest

•• of outstanding interest

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Table 1.

Visual synopsis of CF animal models discussed in this review as referenced to current CF research priorities (CFTR restoration, mucus biology, infection, inflammation, and GI disease). Green check mark indicates documented use through published reports (selected references are provided), red cross mark indicates untested or unsuitable application (for example, absence of spontaneous phenotype), blue question mark

indicates applications under development or restricted to a particular variant of the animal model (for example, humanized variant).

	CFTR Restoration						GI disease
	Gene Therapy	Read-Through Rx	HEMT Response	Mucus biology	Infection	Inflammation	Intestine Pancreas Liver
Pig 	✓ 57	?	✓ 6	✓ 76	✓ 92	✓ 92	✓ ✓ ✓ 6,122,123
Ferret 	✓ 52	?	✓ 11,12	✓ 77	✓ 11,91	✓ 91	✓ ✓ ✓ 11,118,124
Sheep 	?	?	✓ 20	?	?	?	✓ ✓ ✓ 20,21
Rat 	✓ 56	✓ 15	✓ Humanized 65, 66	✓ 13,14	✗ spontaneous ? challenge	✗ spontaneous ? challenge	✓ ✗ spontaneous ✗ spontaneous 13
Mouse 	✓ 55	✓ 62	? humanized	✓ 26,88-90	✓ 27, 93-95	✓ 26, 27, 105-107	✓ ✗ spontaneous ✗ spontaneous 115
Rabbit 	?	?	✓ 23	✗ spontaneous ? challenge	✗ spontaneous ? challenge	✗ spontaneous ? challenge	✓ ✗ spontaneous ✗ spontaneous 23
Zebrafish embryo	✗	✗	✗	✗	✓ 19, 98	✗	✗ ✓ ✗ 18
Drosophila 	✗	✗	✗	✗	✗	✗	✓ ✗ ✗ 22,130
Xenopus Laevis tadpole	✗	✗	✗	✓ 34	✓ 35	✗	✗ ✗ ✗