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斑马鱼癌症模型在肿瘤精准医疗中的研究进展

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[摘 要] 在肿瘤精准医疗中，主要采用两种方法来预测临床相关的肿瘤行为(如治疗响应和药物耐药性的出现)：基于患者样本基因组、转录组、表观基因组和/或蛋白质组学进行分析推断，以及在个体化癌症患者“替身”中进行表型研究。后一种方法依赖于小鼠体内异种移植模型和体外类器官癌症模型或2D细胞培养。患者源性异种移植免疫受损成年斑马鱼品系的建立，以及基于斑马鱼幼鱼异种移植模型药物反应表型测试的第1项临床试验的开展，使斑马鱼这种小脊椎动物应用于精准医疗领域的前沿。

[关键词] 肿瘤精准医疗；斑马鱼；转基因癌症模型；癌症异种移植模型

Research progress of zebrafish patient avatars in precision oncology

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Abstract In precision cancer medicine, two major approaches are used to predict clinically relevant tumor behaviors (such as treatment response and the emergence of drug resistance): analysis and extrapolation based on genome, transcriptome, epigenomics, and/or proteomic of patient samples, and phenotypic studies were performed in individualized models of patients with a cancer. The latter strategy relies on xenograft models in mice and the cancer models of organoids or 2D cell culture in vitro. The establishment of immunocompromised adult zebrafish models of patient-derived xenografts and the development of the first clinical trial based on the drug response test of zebrafish larvae xenografts make this vertebrate to be applied in the frontier of precision medicine.

Keywords precision oncology; zebrafish; transgenic cancer models; cancer xenotransplantation model

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越来越多可供选择的治疗方案，为需要系统治疗的癌症患者带来了希望。除了化疗，各种各样的新型靶向疗法以及最近的免疫治疗药物已经进入临床开发或已获得注册批准[1]，这也给肿瘤科医生带来了新的挑战。如今新药的开发速度超过了那些为确定药物剂量、毒性、治疗方案而开展的漫长而复杂的试验的进度。近年来，转基因[2]和异种移植模型斑马鱼癌症“替身”模型[3-4]，凭借其规模、成本、时间和饲养条件多样性[5]的内在优势和高通量自动化测试的潜在优势[6-7]，逐渐进入精准肿瘤医疗药物反应研究领域。

1 斑马鱼作为癌症“替身”的研究进展

1.1 稳定的转基因癌症模型

人类器官特异性遗传程序和癌症相关基因保守性[8]、端粒生物学与人类相似性[9]、单细胞阶段显微注射可操作性使斑马鱼迅速成为体内模拟癌症有前景的生物[10]。2003年，通过将小鼠基因Myc开放阅读框置于斑马鱼rag2启动子的控制下，斑马鱼T细胞急性淋巴细胞白血病(T-ALL)癌症模型[11]首次建立并报道。该模型为斑马鱼癌症建模提供了理论证明，也为开发斑马鱼其他肿瘤模型奠定了基础，包括2004年的胰腺神经内分泌肿瘤[12]、2005年黑色素瘤[13]、使用Cre介导重组模型以及化学诱导模型[14-16]，以及此后诞生的许多其他模型(图1)。据文献报道，大多数人类癌症，特别是实体瘤中，肿瘤形成需要多种遗传学因素驱动，包括致癌基因的激活和抑癌基因功能的缺失[17-18]。因此仅基于致癌基因表达的斑马鱼模型通常显示出较差的渗透性，这大大限制了斑马鱼模型的应用[12,19]。据报道，第1个黑色素瘤斑马鱼模型利用人类黑色素瘤中发现的最常见的驱动突变$BRAF^{V600E}$，在携带tp53肿瘤抑制基因[20]失活突变的斑马鱼中注射mitfa，即$BRAF^{V600E}$构建体，可形成恶性黑色素瘤[13]。但像一些转基因斑马鱼癌症模型一样[11,13,21-22]，具有一些限制因素，例如维持带有多个转基因的稳定品系非常困难，可组合的转基因数量有限，内源性斑马鱼肿瘤抑制基因的敲除是系统性的而非组织特异性的，人类致癌基因的转基因表达可能没有生理表达水平。基因组编辑技术将有助于克服部分局限性。

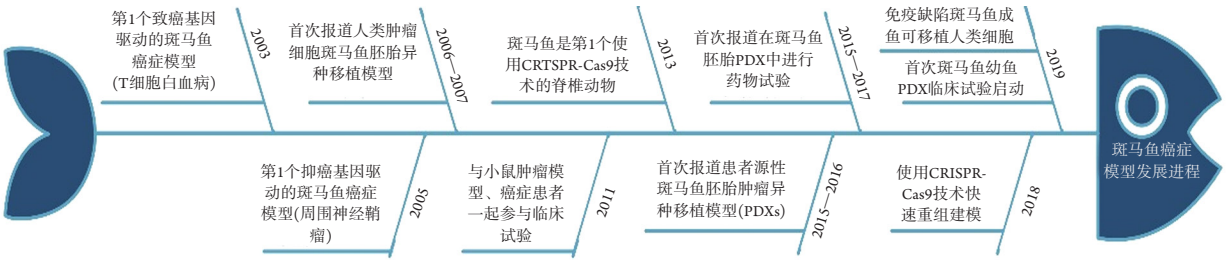


图1 斑马鱼癌症“替身”关键进展事件的时间表

Figure 1 Timeline of key developments in zebrafish cancer avatars

PDX：患者来源的异种移植模型；zPDX：患者来源的斑马鱼异种移植模型。

PDX: patient-derived xenograft; zPDX: zebrafish patient-derived xenograft.

1.2 快速基因重组建模

CRISPR-Cas9系统能实现肿瘤抑制基因的快速整合失活，从而无需稳定的转基因品系。通过复用靶向不同基因的向导RNA(gRNA)，大大增加了组合建模的潜力，为建立体内人类癌症模型创造了新机遇[23]。据报道[24]，如同在小鼠中一样，CRISPR使快速建立敲除斑马鱼品系成为可能。利用Tol2转座子技术[25]，将斑马鱼U6启动子驱动的gRNA与cas9序列组装，在组织特异性斑马鱼启动子控制下，开发出CRISPR载体用于组织特异性基因失活[26]。通过设计靶向斑马鱼肿瘤抑制基因载体，黑色素细胞特异性表达人类癌基因，比如BRAF、NRAS或KIT致癌基因突变结合cdkn2a、tp53、ptena或ptenb基因失活，快速而稳定地建成人类黑色素瘤模型(图2A)。在具有一个可靠组织特异性斑马鱼启动子的前提下，该技术可实现在其他器官中组合癌基因表达和肿瘤抑制基因失活。例如，有研究[31]表明：通过镶嵌CRISPR方法，Hedgehog通路中patched 1(ptch1)基因突变会增加T-ALL发作。载体在F0代中的镶嵌表达足以产生肿瘤，从而不需要建立稳定的转基因品系。局限就是从出生时就需引入驱动基因突变，因此可能会诱发发育缺陷。解决该问题的一种方法是将整合载体直接递送到成年斑马鱼的体细胞组织中，例如最近报道的电穿孔法[32]。该技术可将几个载体一起注入以便对更复杂的基因型进行建模。过表达和CRISPR载体也可以

同时使用，以较高通量来研究癌症驱动因素的相关性。上述建模方式可使斑马鱼成为体内大规模研究肿瘤遗传学的主要模型。

2 斑马鱼癌症异种移植模型

斑马鱼幼鱼透明，casper品系在成鱼期间通体透明，易于进行荧光标记细胞的追踪；繁殖能力强，饲养和维护成本较低；易于显微注射或批量操作(图2B)，可进行大规模和高通量细胞移植研究，而这些在免疫受损的小鼠模型中很难执行[33-40]。这些特征使斑马鱼成为理想的异种移植受体。斑马鱼癌症模型与大规模基因筛选和药物发现平台结合，为研究肿瘤克隆进化和异质性、治疗耐药性、侵袭和转移、造血和干细胞移植提供了有价值的视角[3,34,38,40-44]。

2.1 斑马鱼幼鱼的异种移植模型

斑马鱼的获得性和先天性免疫系统与小鼠和人类高度保守[45]。在斑马鱼中，先天免疫在受精后的第1天就开始出现，而获得性免疫在受精后2~3周成熟。幼鱼在早期发育过程中免疫能力低下，使人或小鼠细胞异种移植和短期存活成为可能，而无需额外处理免疫系统。另外野生型斑马鱼幼鱼在形成色素沉着之前视觉上清晰透明，使得移植细胞的单细胞分辨率成像、分析和定量可实现，这是优于其他移植模型的一个独特优势。但是同时也具有一些局限性：首先，幼鱼中移植细胞被获得性免疫系统排斥之前大约有7 d的短窗口期；其次，斑马鱼幼鱼体型较小限制移植细胞数为100~200；第三，大多数幼鱼受体的异种移植实验是在37 ℃下进行的，在此温度下，有研究报道移植的人类细胞的增殖速率或形成肿瘤块的方式与免疫受损的NSG小鼠或人类患者不太一样[3-4]。

2.2 成年斑马鱼异种移植模型

免疫缺陷斑马鱼模型可将患者来源的癌细胞异种移植到成年斑马鱼受体中。放射或化疗可短暂消融动物免疫系统使成年斑马鱼中异种移植细胞长期移植和存活[46-47]，如γ射线照射、类固醇药物地塞米松[46,48]。但是，化学免疫抑制引入实验变异性，与被测药物相互作用，可能混淆实验结果。

为克服上述限制因素，Yan等[4]建立了一种成年斑马鱼异种移植模型，该模型在透明casper斑马鱼中引入prkdc和il2rga纯合失活突变，使人和小鼠细胞得以稳固、长期植入和增殖。该类免疫缺陷斑马鱼是透明的，缺乏T细胞、B细胞和自然杀伤细胞，可以在37 ℃下存活。斑马鱼生存能力强，随着时间的推移，斑马鱼可以很好地耐受该温度。适应后的斑马鱼可稳固植入大量人类癌细胞系和PDXs超过28 d。与幼鱼移植受体相比，除了更长的实验窗口期和人类生理温度外，成鱼的另一个主要优势是可以植入数量更多的移植细胞(每条成鱼可达200万个细胞)。其生长动力学、细胞增殖和凋亡率与在NSG小鼠中相同。

3 在斑马鱼癌症“替身”中进行药物测试

斑马鱼转基因和异种移植肿瘤模型可作为患者“替身”进行多种临床药物试验，相应的临床决策可以帮助患者找到最合适的疗法(图1)，并且与其他体内患者特异性“替身”相比，斑马鱼“替身”显著增加药物测试的规模。

3.1 斑马鱼的给药途径

文献[2-3]表明：斑马鱼药物递送方法包括：一是将药物直接加入水中，浸没幼鱼或成鱼；二是成鱼通过腹膜内注射或口服管饲给药[4,28-29](图2C)。与浸没法相比，成鱼口服管饲或腹膜内注射给药可以严格控制剂量，可通过质谱法评估血浆样品的药代动力学；管饲和腹膜内注射可有效递送低溶解度的小分子和抗体。人、小鼠和斑马鱼之间的药代动力学具有差异，根据实验研究，已确定出口服管饲和浸没法间的剂量转换计算方式[3,4,28,30]。此外，成年斑马鱼PDX(zPDXs)中化疗药物(替莫唑胺)和靶向药物(奥拉帕利)管饲给药，其血液药代动力学特征与人类和小鼠非常相似[4]，因此药代动力学并不是斑马鱼“替身”的跨物种障碍。

3.2 临床响应确定

通过测量磁共振成像或计算机断层扫描成像数据中最大肿瘤直径变化来确定患者对癌症治疗的临床响应。根据大小变化，依据RECIST标准对患者响应进行分类[49]。在斑马鱼患者“替身”中，有研究报道了多种方式测定药物响应：1)在透明受体中对未标记的肿瘤细胞直接成像(特别是天然色素瘤，比如黑色素瘤)[28]；2)肿瘤大小的荧光成像[4]；3)将癌细胞暴露于活性荧光染料[3-4]；4)无创超声检查[50]。根据RECIST标准，细胞数量(幼鱼zPDX)和肿瘤表面积(幼鱼和成鱼zPDXs)已用于评估肿瘤响应[30]。这些方法与体内高通量斑马鱼筛选平台相结合，可以在单细胞分辨率水平上实时评估药效。自动化注射系统和斑马鱼幼鱼自动化成像系统的出现有利于其标准化和临床应用[7]。

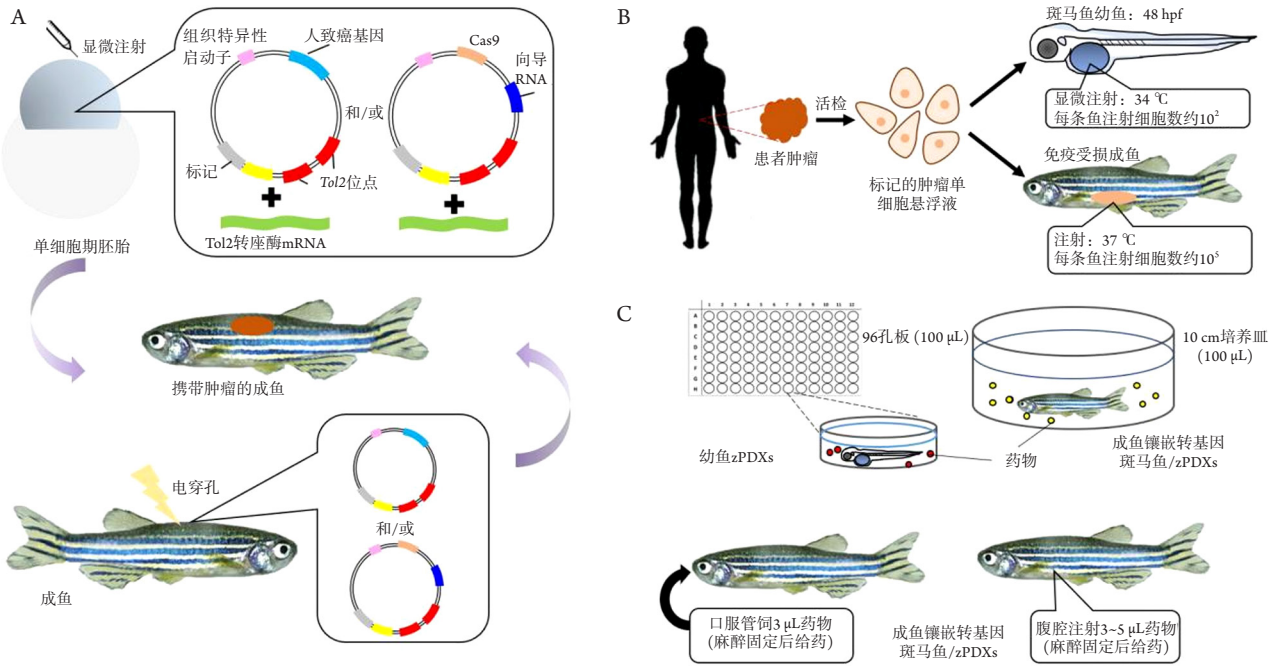


图2 斑马鱼癌症模型的建立和斑马鱼给药方式[26-31]

Figure 2 Generation of zebrafish avatars and drug administration in zebrafish[26-31]

(A)胚胎显微注射或成鱼中电穿孔法建立镶嵌转基因斑马鱼模型。(B)患者源性斑马鱼异种移植模型的建立。(C)斑马鱼的给药方式。浸没在药物溶液中[24孔板每孔可容纳10~20个24 hpf(受精后小时)胚胎，96孔板每孔可容纳1个胚胎，每个直径10 cm培养皿可容纳2条成鱼]，口服管饲法或腹腔注射法用于处理成鱼。MS-222单独或与异氟烷联合用来麻醉并固定斑马鱼。有报道的注射药物可耐受范围为3~5 μL[4,28]。

(A) Mosaic transgenic zebrafish can be created using embryo microinjection or electroporation in adult fish. (B) Establishment of patient-derived zebrafish xenograft. (C) Drug administration in zebrafish: Submersion in drug solution is used to treat larval zebrafish (10–20 per 24 hpf embryos per well in a 24-well plate or one embryo per well in a 96-well plate) or adult avatars (two adults in a 10-cm Petri dish). Oral gavage peritoneal injection can be used to treat adult avatars. Zebrafish are anaesthetized and immobilized using MS-222 alone or in combination with isoflurane. Approximately 3–5 μL for medication injected is the tolerable range reported[4,28].

3.3 可测试的药物类型

“替身”模型与生物体靶标保守性之间的生物学差异直接影响药物评估(图3)。多个研究报道了在斑马鱼中已测试过多种抗癌药物，包括多种化疗药物、小分子抑制剂(如达沙替尼)和抗体药物(如贝伐珠单抗和西妥昔单抗)[3,51-62]。针对肿瘤微环境的靶向治疗依赖于更精确的靶标保守性，更适合于转基因模型，但是针对其他基因的靶向治疗或合成致死疗法的成功取决于靶标保守性[63-64]。但zPDX模型中缺乏患者特异性的免疫肿瘤微环境，缺乏跨物种的靶标保守性(如免疫检查点PD1、PD-L1和CTLA4序列的保守性)使在斑马鱼“替身”中评估患者对靶向免疫肿瘤微环境药物响应充满挑战。幼鱼中另一个混淆因素是许多抗癌药物对斑马鱼幼鱼受体本身存在潜在毒性作用。有研究[65-69]认为，幼鱼发育速度快，观察到的肿瘤缩小也可能是毒性对受体本身的间接影响，毒性作用会削弱受体支持癌细胞生长的能力。

3.4 响应或耐药预测

斑马鱼“替身”对肿瘤精准治疗的价值可归为3方面：预测能力、实验规模及实验持续时间。研究[70]表明：耐药性通常分为原发性或内在性耐药(患者从未对药物产生过响应)和继发性或获得性耐药(患者最初对药物敏感，后续会产生耐药性)。例如，在BRAF或KIT驱动的黑色素瘤中，spred1失活的快速重组转基因斑马鱼模型中检测到原发性耐药。浸没法治疗成年转基因斑马鱼和黏膜黑色素瘤患者的靶向治疗，证明这种功能缺失驱动了KIT酪氨酸激酶抑制剂的耐药性。机制研究进一步表明了MEK和KIT抑制剂联合治疗的潜在疗效。鉴于发现药物原发耐药性的暴露时间短，幼鱼和

成鱼zPDXs都是解决此问题的合适模型。以肿瘤活检样本建立的幼鱼zPDX与肿瘤类器官模型十分相似[71]，可能在手术前的新辅助治疗中特别有用，有助于快速选择有效的化疗方案。

另一方面，有研究[72]发现：这种短期幼鱼移植试验，每个受体接受的细胞数量有限，不太可能是继发性耐药的良好预测模型。因此推测，成年zPDX的窗口期越长且细胞数量越多，将更好地预测由小部分耐药基因突变的扩展或转录适应而引发的继发性耐药机制。

4 癌症“替身”之间的比较

不同癌症“替身”在生物学和后勤(饲养成本、条件)等方面均具有不同的优势和局限性，这使得它们能适合于各种临床前药物开发或个性化药物测试。一般来说，限制转基因癌症模型在精准肿瘤学中应用的关键因素是建立“替身”所需的时间和为了模拟癌症基因组复杂性而进行的遗传学组合。由多项研究可归结出斑马鱼、小鼠和类器官癌症“替身”模型的比较[4,27](图3)。

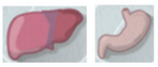
癌症模型	试验时长	花费[a]	后勤条件[a]	每个受体所需细胞数量	药物筛选通量[b]	癌细胞内源目标保守度	患者肿瘤微环境保守度	药代动力学和剂量优化
 镶嵌转基因斑马鱼	数周至数月	低	中	NA (仅患者肿瘤测序时需要)	中	驱动基因100% 其他可变	无	有
 幼鱼zPDXs	5~7天	低	低	10^2	高	高	无	无
 成鱼zPDXs[c]	数周至数月	低	中	10^5	中	高	低或无	有
 体外人类3D器官	1~2周	低	低	10^2~10^3	很高	高	无	无
 鼠PDXs	数周至数月	高	高	10^5~10^6	低	高	低或无	有

图3 个性化癌症模型间的比较

Figure 3 Comparison of personalized cancer avatars

NA：不适用；PDX：患者来源的异种移植模型。a：虽然费用和饲养规则因国家和机构而异，但先前在北美和一些欧洲国家报道的饲养费用和条件表明，在物理空间要求(每1.2升，约12只成年斑马鱼vs 1只成年鼠)和每天的成本(每只3.5升鱼缸为美分级vs每只老鼠笼为美元级，每只老鼠约1.05美元，每只成年斑马鱼约0.01美元)上，斑马鱼比老鼠低至少一个数量级。b：兼顾筛选持续时间与临床决策，药物筛选通量数量级(数天至数周)划分为低(1~5个化合物)、中(10~50个化合物)、高(约100个化合物)，很高(约1 000个化合物)。c：该数据基于前人研究，需要进一步研究成年斑马鱼PDXs种不同肿瘤类型的植入率、试验时间和细胞数。

NA: not applicable; PDX: patient-derived xenograft. a: While the cost and rules of feeding a zebrafish and a mouse differ significantly because of the diverse requirements in countries and institutions, the cost and housing rules in North America and some European countries reported previously suggest that the requirements of husbandry physical footprint space (about 12 adult zebranfish per 1.2L VS a mouse per 1.2 L) and the cost per day (per 3.5 L of a fish tank for cents vs per mouse cage for dollars; about 1.05 dollars per mouse and about 0.01 dollar per an adult zebrafish), which are at least an order of magnitude lower for zebrafish than for mice. b: Considering the duration of screening and clinical decision-making, the drug screening throughput orders of magnitude (days to weeks) are the following: the low (1–5 compounds), the medium (10–50 compounds), the high (about 100 compounds), and the very high (about 1 000 compounds). c: The data are based on previous studies. Engraftment rates, assay duration and cell numbers need further studying.

5 结语

自2011年以来，由于后勤方面的限制，小鼠PDX协同临床试验除了概念验证研究以外进展甚微[73-75]。因此斑马鱼癌症“替身”在模型开发的规模、成本和速度以及自动化潜力方面具有独特的优势。

就个性化转基因“替身”而言，时间问题(测序、数据分析和载体建立需要1~4周，加上肿瘤潜伏期长达数周至数月)限制了它们的优势，无法为临床决策提供依据。但它们仍是阐明癌症遗传学和新药开发中最有用的临床前模型。成本的不断降低、DNA测序和合成的速度、适用性的提高，以及基因工程技术的改进，都能帮助建立起更快、更复杂的转基因斑马鱼模型，来研究患者可能出现的治疗反应。研究[45]表明由于斑马鱼异种移植技术已经成熟，并且也有了建立幼鱼和成年zPDX模型的强有力的研究支持，对于该领域而言，转向临床、进行临床研究至关重要。幼鱼模型在以速度和短期响应为目标的临床研究中具有潜在价值，例如术前新辅助化疗。除了药物反应预测外，还可利用幼鱼斑马鱼PDX评估癌症转移行为，但是还需要进一步的研究来评估胚胎发育中的迁移是否会造成人类癌症的转移风险或器官偏好性。已有的小鼠PDX和成年zPDX之间生物学和预测等效性的证据，加上较低的成本和繁殖力优势，以及斑马鱼模型药物筛选通量较高的优点，使斑马鱼成为肿瘤精准医疗中有前景的生物。

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