CRITIQUE & DEBATE

MOLECULAR BIOLOGY & GENETICS

On the low reproducibility of cancer studies

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Earlier reports suggest that close to 90% of cancer biology publications are irreproducible (2). The low number has recently been corroborated by 5 detailed replication studies in eLife (1). While the irreproducibility is often attributed to human factors (1), which are remediable, the reason might be biological and the irreproducibility is intrinsic to such studies. The low reproducibility, reflecting the diversity in the evolutionary pathways of tumorigenesis, will likely impact clinical strategies significantly.

1. Background

Reproducibility is the foundation of experimental science. While there are many factors afflicting different fields of inquiry, and to various degrees (2), cancer biology studies appear to stand out. In an earlier report, only 6 of 53 published findings in cancer biology could be confirmed (3), a rate approaching an alarmingly low 10% of reproducibility. According to the report, the low rate is a common opinion in the pharmaceutical industry.

The low rate is of particular concern because these studies are generally published in “high-impact” journals, thus having real consequences in both clinical practice and basic research. In this critique, we first review the recent updates on the extent of reproducibility. Second, the reasons underlying the low reproducibility are explored. While concerns about the low reproducibility are often expressed in terms of human factors (1, 3-5), there are deeper biological reasons. Third, a distinction should be made between studies that should be reproducible and those that are intrinsically irreproducible. Fourth, suggestions are made to cope with the irreproducibility issues.
2. Updates on the reproducibility investigation - The RP:CB project

The Reproducibility Project: Cancer Biology (RP:CB) was launched to address the issue rigorously (6). RP:CB has selected 30 cancer studies from high-impact journals for reproducibility investigation. Because failure to reproduce is most likely due to technical factors, ruling out these obvious reasons is the salient contribution of RP:CB. Prior to the actual replication is a phase of Registered Reports, which outline the replication designs. The replication studies themselves only began after the designs had been reviewed and approved. Since both sides agree on the principle of reproducibility, the challenge is to assure that the materials and methods can be accurately replicated.

Now, the first batch of reports has been released (7). Reproducibility here means “actionable and repeatable therapeutic strategy” (3), as each of the original studies has a therapeutic proposal. According to the eLife editorial (4), two studies are “reproducible in important parts”, one being “not reproducible” and the remaining two being “un-interpretable”. Another news report characterizes the overall picture as “muddy” (5). We found these readings of the replication results to be overly cautious. In a more conventional assessment (see Table 1 for a summary), 4 of the 5 original studies should be classified as “not reproducible” and one being “un-interpretable”.

We shall start with the two reports which eLife editorial considers un-interpretable due to irreproducible controls. As the failure to reproduce the control is no different from the failure to reproduce the experiments, we reclassify the two cases as “not reproducible”. In Berger et al. (8), mutations in the PREX2 gene are found to be common in human melanomas (15 out of 107). Furthermore, the introduction of a PREX2 mutation into human melanocytes was reported to substantially reduce the tumor-free survival in the xenograft mice. In the replication study, Horrigan et al. (9) (see also Davis (10)) cited recent studies that fail to support the prevalence of PREX2 mutations in human melanomas. In addition, Horrigan et al. failed to corroborate the reduction in tumor-free survival caused by PREX2 mutation because the controls without the mutation die just as rapidly (1 week), in contrast with the original report of a 9-week median survival in the control.

In the other study, Willingham et al. (11) suggest that the CD47 protein is over-expressed on the membrane of most cancer cells. Since CD47 signals to macrophages to withhold attack, blocking CD47 reduces the mass of orthotopic breast tumors by ~10 fold in immune competent mice that have been injected with MT1A2 mouse cells, relative to the control. In the replication by Horrigan (12), the tumor sizes are curiously reversed between the CD47 and IgG (control) treated mice. Horrigan noted that the tumor mass is highly variable, often with a 5 fold difference in the same setting. Such high variability is common is tumor evolution but is often treated as noises. It should be noted that Willingham et al. obtained similar results by transplanting human tumor cells to immune compromised mice. Horrigan reported that the xenograft experiments have been reproduced only in some follow-up studies.

The one study deemed not to be reproducible is Sugahara et al. (13) who showed an increase in drug permeability when tumors are treated with the iRGD peptide. In their study, tumors grow from xenografted prostate cancer cells. Mantis et al. (14) could not reproduce the results but they report some successful replications by other studies (15-17). While Sugahara et al.’s study was the only study whereby the experiments could not be reproduced, it is possible that either the control or the experiment can be more variable. Thus, it seems curious to consider one type of irreproducibility more serious than the other.
For the remaining two studies declared “essentially reproducible”, one (18) would not be considered reproducible under most circumstances. In Sirota et al., the bioinformatic analysis of drug applications on cell lines has led to the identification of cimetidine as an effective agent against lung adenocarcinoma cells. While the replication by Kandela et al. (19) observes the same trend, the difference is not significantly different from the control. The reason, as noted by Dang (20), is that the effect of cimetidine reported by Sirota et al. is too weak to be considered biologically significant. Overall, the variance, in comparison with the small difference in mean, makes it difficult to justify the conclusion of cimetidine being an effective new drug against lung cancer.

The only reproducible study in the set of 5 studies is that of Delmore et al. (21). In the original study, the molecule (+)-JQ1 is reported to down-regulate MYC transcription and reduces the burden of multiple myeloma tumor, resulting in the improved survival of the xenograft mice. While the results were successfully replicated by Aird et al. (with some variations) (22), a negative control using an enantiomer (-)-JQ1, which does not impact MYC transcription, shows the same biological effect as (+)-JQ1. Given that the negative control also yields the (unexpected) benefit, we suggest the original study to be un-interpretable.

3. Causes of irreproducibility

The five replications corroborate the earlier report of reproducibility at a rate of 6 out of 53 (3). While previous reports hint of technical (or even ethical) lapses (3-6), the factors cited are common across biological disciplines. Furthermore, given the care invested in RP:CB, the replication efforts should be quite adequate. We therefore seek biological explanations below.

Whether any experimental study can be replicated depends on the measurements being reproduced. In coin tossing, seeing the same side five times in a row will be reproducible no more 10% of the time. In cancer biology, reproducibility would mean that tumor progression corresponds to highly constrained courses, akin to tissue development. However, if tumor progression follows evolutionary trajectories, the outcome may be highly variable. The course of evolution is often a multi-step process requiring a suite of genetic changes, each of which is governed by stochastic factors including mutation emergence, random drift and divergent selective pressures. Since each step is contingent on the previous steps taken, divergent outcomes may result from even a small deviation in an earlier stage.

In his book “Wonderful Life”, S. J. Gould (23) raised the issue of the reproducibility of evolution itself. He wondered if the same evolutionary trajectory would be followed had “the tape of life” been rewound (see also Conway Morris (24)). “Rewinding the tape of life” back to the time of Cambrian explosion is of course mere fantasy but there are indeed evolutionary processes that are continually reiterated. The best example may be the evolution of cancers (25-28). It is hence curious that the word “evolution” does not appear in the RP:CB registered/replication reports, editorials and commentaries.

Reproducibility of evolution would be equivalent to convergent evolution in which a dominant pathway is repeatedly taken. In convergent evolution, the distinction between phenotype and genotype is crucial. Phenotypic
convergence is common in natural populations. Similarly, morphological convergence is a basis on which pathologists define malignancy. The central issue is genotypic convergence - whether the genetic changes underlying the phenotypes are themselves convergent. With constant references to somatic mutation, gene expression and target therapy, cancer biology publications apparently consider genotypic convergence plausible.

In this backdrop, the TCGA project (the cancer genome atlas; (29-31)) attempted to identify genes commonly mutated in tumors. The results show that genetic convergence is much less frequent than hoped for (29). For example, across 12 cancer types, only two genes are mutated in more than 10% of cases – TP53 and PIK3CA; the former is an outlier in the human genome (32, 33) and the latter is a very large gene. The number of frequently mutated genes (in >10% of cases) for a given type of cancer is generally around 10 (29, 30). With such low genic convergence, two cases of the same cancer type usually have few mutated genes in common, or may share no mutated genes at all. These observations suggest that, from a very similar starting point (two human beings), the evolution of cancer usually takes different courses. Even cancer cells from the same starting point (within the same person) would continue to diverge, leading to substantial genetic diversity (34, 35) and variable responses to therapeutic treatments (36).

The TCGA results have their parallel in natural populations. While genotypic convergence may be observed for highly specialized traits such as echo-location (37), it is nevertheless rare. For adaptations that do not involve highly specialized constructs, diverse molecular mechanisms may operate and genotypic convergence is not expected. For example, human populations living in the high altitude of Tibetan, Ethiopian and Andean Altiplano plateau have different genetic mutations for hypoxic adaptation (38). Recently, the search for molecular convergence has expanded to finding signals in the entire genome (37, 39-44). Again, even with the aid of multiple genomes in the same environment, molecular convergence is uncommon and the signals rarely exceed those of the background noises.

Reproducibility of cancer progression and convergent evolution in organisms are nevertheless observable but the conditions are stringent – when there is a dominant evolutionary pathway leading to an end state. For example, when the organisms are genetically simple (e.g. viruses), there would often be few genetic solutions. Alternatively, if the selective pressure for specific genetic changes is strong (e.g. (39)), then convergence is a likely outcome. Wu et al. (2016) speculate that the selective pressure may be particularly high in “liquid tumors” where cells with a proliferative advantage can spread rapidly and widely. Indeed, chronic myelogenous leukemia (CML) remains one of the best examples of cancer convergence at the genic level with the BCR-ABL translocation being a diagnostic feature (45, 46). In general, when we take into account the multi-phenotypic and multi-genic nature of tumor evolution (see Hanahan & Weinberg 2011 on cancer hallmarks (33) and Kandoth 2012 (29) on cancer driver genes) as well as the complexity of mammalian genome, molecular convergence in cancer progression would likely be the exception rather than the rule (26).

As TCGA reveals the low convergence in real-life tumorigenesis, one might still expect a high level of convergence in mouse models when most conditions are under control. Now, the RP:CB studies have cast doubt on the predictability of evolution even in simple models. In the 5 RP:CB reports, cells from cancer cell lines are transplanted into mice, as xenografts or autografts. In these studies, experimental (E) and control (C) samples are collected, designated (E1, C1) for the original studies and (E2, C2) for the replications. Tumor growth in each mouse is the culmination of two evolutionary processes. First, the cell populations have been evolving prior to
transplantation (47). Second, these cells subsequently evolved as xeno(auto)-grafts into tumors. While the second stage is widely discussed (see Wu et al. (26) for references), the first stage has been neglected even though cell lines do evolve continuously. This first stage is reminiscent of the classic “Luria/Delbruck fluctuations” (48).

Results from E1, C1, E2 and C2 are all conditional distributions (Fig. 1). The final analysis of RP:CB rests on the comparisons between (E1-C1) and (E2-C2). In those replication reports that fail to reproduce the original results, C1 and C2 have not evolved along the same path in two reports, and E1 and E2 have evolved divergently in one (see Table 1). In another report, E1-C1 is too small to be biologically or statistically significant.

Tumor evolution in RP:CB may be sketched by a simple genetic model (Fig. 1) which frames evolutionary pathways as conditional probabilities. Each stage of evolution is conditional on prior steps of evolution via segregation of existing polymorphisms and emergence of de novo mutations. A slight difference in the early stage may pave the way for a much greater divergence at a later time. In Stage 2 of Fig. 1, the two replications overlap little in their trajectories and very different tumor phenotypes emerge as a result. A more realistic model than that of Fig. 1 will likely yield even more diverse patterns. We suggest that cancer biology studies should develop explicit evolutionary models, rather than assume simple and reproducible outcomes.

4. What studies are, or are not, reproducible?

The RP:CB reports give a glimpse of what may or may not be reproducible (7). If the phenotype being assayed does not evolve in the course of the experiments, the reproducibility is generally high. For example, the treatment of cells with the chemical JQ1 leads to the reproducible down-regulation of MYC transcription (21, 22). Similarly, Horrigan (12) was able to reproduce the toxicity effect resulting in mild anemia in normal mice, whose tissues have not evolved.

In the evolution of tumors, the reproducibility would be a function of the number, strength and length of the evolutionary pathways. The TCGA data suggest that the number of genetic pathways for tumorigenesis must be quite large. As the number of steps in each pathway increases, the number of possible alternatives increases exponentially, rendering many observations irreproducible. We should note that the sort of contingent evolution depicted in Fig. 1 results in variable outcomes that may be difficult to capture by increasing the sample size.

Facing the diversity of pathways, cancer biology studies often attempt to isolate cases sharing part of their pathways; for examples, lung cancer cases sharing the mutated EGFR gene. These cases may indeed show robust and more reproducible outcomes in responding to EGFR inhibitors (49). However, such partially-defined genetic pathways are still quite diverse and irreproducible results, as in drug resistance, are not uncommon.
5. Conclusion

It is curious that the RP:CB project (4, 6) together with the earlier reports (3) are met with near total silence. Perhaps, the prevailing view that the low reproducibility is attributable to human factors (1, 5) does not call for intellectual discourses. This view, unsupported by any evidence, may have obscured the more fundamental reason of irreproducibility. In the evolutionary perspective, the low reproducibility is intrinsic to such studies because tumorigenesis does not usually traverse the same evolutionary pathway.

Reproducibility is nevertheless the central tenet of cancer biology, which assumes convergent pathways with relatively well-defined genetic changes. Thus, in basic research, the genetic changes are identified and, then, therapies are developed to target these changes. The continual evolution of the underlying genetic architecture means that mutations are “moving targets”, both between and within individuals. In this perspective, target-gene therapy operates against the evolutionary rules.

Traits that do not evolve or evolve slowly may be better targets. Indeed, the evolution of drug resistance continues to be a major impediment in targeted cancer therapy. The efficacy of the top 3 monoclonal antibody drugs, bevacizumab, trastuzumab and rituximab, are instructive (50). Bevacizumab and trastuzumab targeting VEGF and HER-2, respectively, have limited success (50, 51). In contrast, rituximab that targets CD20 on the cell surface of all pre-B cells significantly improves survival in patients with B-cell lymphoma (52). The efficacy of rituximab may be due to the fact that it does not target a product of cellular evolution. Recent strategies that target the basal transcription machinery (53, 54) are compatible with this view favoring non-moving targets.

Finally, many diseases are the (by-)products of evolution in the view of Darwinian medicine (55). Tumorigenesis is different as it is not merely the product of evolution (56); it is the process itself, or evolution in action (57). Lewontin (58) pointed out that the essence of evolution is the variability. The diversity, rather than a standard type, is the subject of interest. In cancer studies, the diversity in evolutionary trajectories is also clinically significant. H. J. Muller remarked that biology has gone too long without the evolutionary thinking at the centennial of the publication of “Origin of Species”. Five decades later, Muller’s remarks remain relevant to the studies of cancers.

Notes:

There have been four additional reports released by eLife (DOI: 10.7554/eLife.25306.001, DOI: 10.7554/eLife.26030.001, DOI: https://doi.org/10.7554/eLife.29747.001, DOI: https://doi.org/10.7554/eLife.30274.001) at or after the time of this submission. The Elife editorial considers three as reproducible in “important parts” and one as reproducible in “some parts”. Among the four studies, all reproducible experiments are carried out on cell lines growing in dish and the time unit for collecting data after treatment is either minute or hour. The only xenograft tumor model examined is in Shan et al. (DOI: 10.7554/eLife.25306.001), which tests the efficacy of I-BET151, an inhibitor of the BET bromodomain, as a treatment for MLL-fusion leukaemia. The experiment takes two months, thus allowing for plenty of mutation and selection events. Not unexpectedly, the replication group failed to reproduce the original finding of increased survival in I-BET151 treated mice. The timescale-dependent reproducibility of cancer research strengthens the evolutionary arguments of our report.
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Table 1 Summary of the first batch of RP:CB studies

| Studies          | eLife editorial | This Study | Comments                                                                 |
|------------------|-----------------|------------|---------------------------------------------------------------------------|
| Sirota et al. (18) | Yes             | No         | Cimetidine slowing down lung adenocarcinoma growth. Reported effect is weak in the original report and the weak effect is found insignificant in replication. |
| Kandella et al. (19) | Yes             | No         | JQ1 binding MYC and slowing down myeloma growth. The experiment is reproducible but a negative control using (-)JQ1 has the same effect on tumor growth. |
| Delmore et al. (21) | Yes             | UI         | Co-administration of iRGD with chemo-agent enhances drug uptake by tumor cells. Neither drug uptake nor tumor growth is reproduced. |
| Aird et al. (22) | No              | No         | Transplanted melanoma cells expressing a mutated PREX2 gene grow faster as tumors, speeding up death. In replication, the control cells have the same lethal effect. |
| Sugahara et al. (13) | No              | No         | Anti-CD47 antibody promotes growth of mouse breast cancer cells by blocking phagocytosis, vis-a-vis the IgG control. The opposite effect is observed in the replication. |
| Mantis et al. (14) | No              | No         | Anti-CD47 antibody promotes growth of mouse breast cancer cells by blocking phagocytosis, vis-a-vis the IgG control. The opposite effect is observed in the replication. |

Yes – reproducible; No – Not reproducible; UI – Un-interpretable
Fig. 1 A model on the pathway diversity in tumor evolution. Each step of the pathway is the realization from a probability distribution that is conditional on the previous steps taken. In this model of 12-loci (a –l), one locus may change in each stage. The vertical bar below indicates the evolvable locus, which may change from, say, e to E (and then to E’, E’’ etc). The locus that actually changes is marked in red. It is assumed that each locus has positive fitness epistasis with the two adjacent loci on each side (e.g., E interacts positively with C, D, F and G). Stage 1 represents cell line evolution and stage 2 represents the evolution of these cells into tumors. Evolution of two populations (replications) is portrayed. The evolved genotypes in stage 2 determine the tumor’s phenotypes (bottom of the figure), which show no overlap between populations. If 10 samples are taken from each replication as shown by the small arrows, the two replications would appear totally irreproducible.