# Doxycycline inducible overexpression systems: how to induce your gene of interest without inducing misinterpretations

**Jolan De Boeck\* and Catherine Verfaillie**
Department of Development and Regeneration, Stem Cell Institute, KU Leuven, Leuven 3000, Belgium

**ABSTRACT** The doxycycline inducible overexpression system is a highly flexible and widely used tool for both in vitro and in vivo studies. However, during the past decade, a handful of reports have explicitly called for caution when using this system. The raised concerns are based on the notion that doxycycline can impair mitochondrial function of mammalian cells and can alter properties such as cell proliferation. As such, experimental outcomes can be confounded with the side effects of doxycycline and valid interpretation can be seriously threatened. Today, no consensus seems to exist about how these problems should be prevented. Moreover, some of the strategies that have been used to cope with these difficulties can actually introduce additional problems that are related to genomic instability and genetic modification of the cells. Here, we elaborate on the above statements and clarify them by some basic examples taken from our personal wet-lab experience. As such, we provide a nuanced overview of the doxycycline inducible overexpression system, some of its limitations and how to deal with them.

## INTRODUCTION

Ever since their development in the early 1990s by Gossen and Bujard (Gossen and Bujard, 1992; Gossen et al., 1995), doxycycline inducible overexpression systems have been powerful and flexible tools to study the function of numerous genes in mammalian cells. Despite their biological ingenuity and elegance, the use of these systems can be quite challenging from an experimental design perspective. Here, we will highlight some of these concerns based on personal experiences in the lab. In the experiment of interest, we assessed the effect of a gene of interest (GOI) on the proliferation rate of in vitro cultured pluripotent stem cells (PSCs), under the hypothesis that no effect would be observed. An overexpression cassette containing the GOI, driven by a doxycycline inducible promoter (Tet-On system), was stably integrated into the genome of

PSCs, followed by an antibiotic selection procedure. Subsequent genotyping did not identify any large genetic aberrations. These genetically modified PSCs (further referred to as iGOI cells, i.e., inducible GOI cells) were cultured for 4 d in the presence or absence of doxycycline, after which the number of cells was determined (and normalized for initial seeding number) by measuring the absorption of a crystal violet dye. Five replicates were obtained by using different passages of the same cell line on five different occasions. For the sake of argument, an abstract representation of the actual experimental results will be shown. In what follows, we will demonstrate how different—apparently correctly chosen—combinations of control and treatment groups can lead to invalid conclusions.

## LEAVING OUT DOXYCYCLINE

One of the fundamental basics of designing experiments is the use of appropriate control groups, which should differ from the treatment group only by means of the applied treatment. A quick search through the literature shows that for doxycycline inducible overexpression systems, the iGOI cells to which no doxycycline is added are often used as the control, and this strategy is even recommended by companies making doxycycline inducible plasmids commercially available (Clontech Laboratories, n.d.). This appears indeed to be the ideal setup, as cells in both groups

(i.e., "iGOI – Doxy" vs. "iGOI + Doxy") come from the exact same maintenance culture. If instead a different batch of cells would be used as control, any observed phenotype could be caused by differences in the (genetic) background of the two cell batches (this is what we will further refer to as "batch effects").

For the example considered here, the former approach revealed that the overexpression of our GOI (i.e., "iGOI + Doxy" treatment) reduced the number of cells relative to the "iGOI – Doxy" condition (Figure 1A), an observation that could easily be interpreted as being the direct consequence of the GOI. Upon reflection, however, we realized that the phenotype might instead be related to the presence of doxycycline.

Indeed, previous studies, dating back to as early as the 1980s (Kroon *et al.*, 1984; van den Bogert *et al.,* 1986a,b), demonstrated that doxycycline per se (and tetracyclines in general) can drastically affect the proliferation of mammalian cells. Although the concentrations in these initial studies were relatively high (i.e., 5–10 µg/ml), more recent publications reported similar effects even for the lower doses that are frequently used for inducible overexpression experiments (i.e., 1–2 µg/ml) (Ahler *et al.*, 2013; Moullan *et al.*, 2015; Luger *et al.*, 2018). Furthermore, these studies highlighted that doxycycline not only alters cell proliferation but can also cause impaired mitochondrial protein homeostasis, activation of the unfolded protein response, reduced cellular oxygen consumption, elevated glucose consumption, cell cycle arrest in $G_1$ phase, apoptosis, and global transcriptional changes. Needless to say, these adverse effects can confound the phenotype of interest and should receive the necessary attention. In the next sections, we will discuss some approaches that are commonly used in published reports to overcome this difficulty and highlight points of caution in interpreting the corresponding results.

## MINIMIZING DOXYCYCLINE CONCENTRATIONS

A straightforward precautionary measure against the side effects of doxycycline is keeping its concentration levels as low as possible (concentrations as low as 100 ng/ml can successfully induce overexpression [Zhou *et al.*, 2006]). Ideally, a dose-response curve should be generated to determine the optimal concentration that induces the GOI while being nontoxic for the cells, where toxicity is typically assessed by measuring cell death. It is crucial, however, to realize that doxycycline can—and likely will—have subtler and potentially unobserved side effects than cell death. In contrast with some other authors (Chatzispyrou *et al.*, 2015), we do not a priori advocate against the use of doxycycline. Nevertheless, we fully agree with the fact that caution should be taken when interpreting experiments in which "+Doxy" and "–Doxy" conditions are compared, even when using low concentrations.

## BEING PRAGMATIC

Admittedly, certain experimental outcomes can be more safely interpreted by direct comparison of "+Doxy" and "–Doxy" conditions than others. If, for example, one would observe an increased proliferation after doxycycline administration, it seems reasonable to believe that this effect is caused by the GOI, as we know that doxycycline should have the opposite effect. As another example, consider the inducible overexpression of a master regulator to direct differentiation of PSCs into neurons. As it is well established that doxycycline itself cannot govern neuronal differentiation, pragmatic reasoning allows us to interpret the differentiated phenotype as being the result of the GOI.
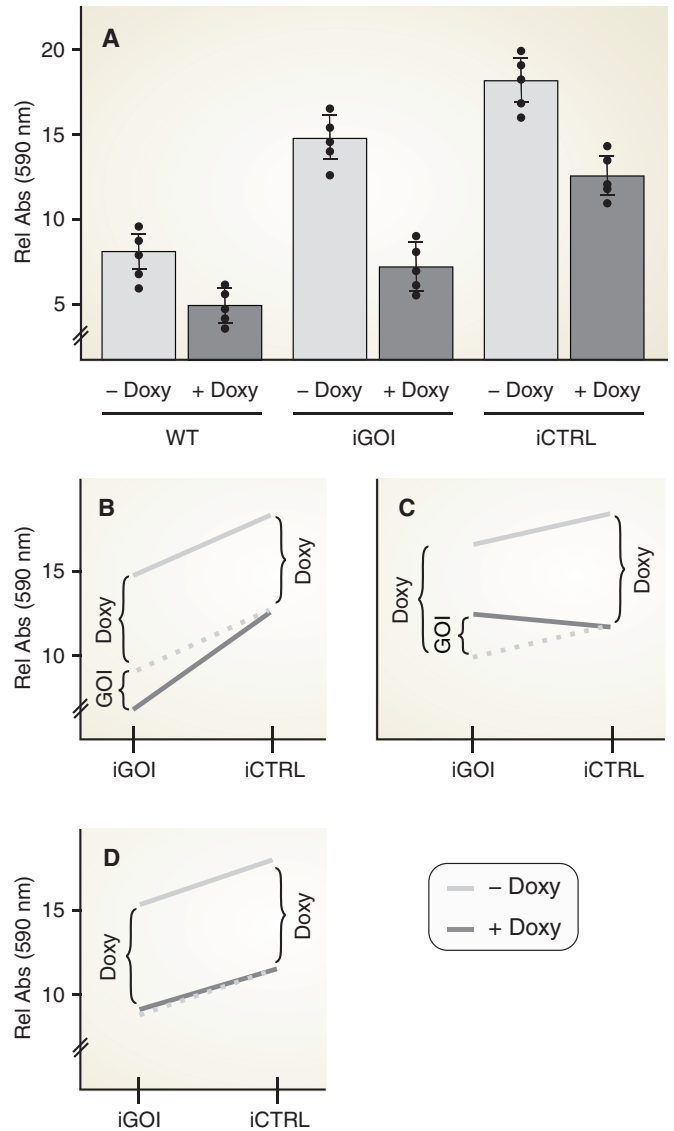


FIGURE 1: Different strategies to analyze doxycycline inducible overexpression experiments. (A) Depending on which control conditions are chosen, the result of the experiment might lead to contradictory interpretations. The five dots per condition denote replicates based on five different passages of a single genetically modified cell batch. Rel Abs = Abs(day 4)/Abs(day 1). See main text for details. Abs, absorption. (B) Interaction diagram (using the heights of the bars in panel A) showing how the difference between "iCTRL + Doxy" and "iCTRL – Doxy" can be used to deduce the effect of the GOI, under the assumption that iCTRL and iGOI cells are equally sensitive to doxycycline (i.e., the dotted line runs parallel to the light gray solid line) or, generally stated, under the assumption that the GOI is the only systematic difference between iCTRL and iGOI cells. (C) A similar analysis as in panel B for different batches of independently generated iCTRL and iGOI cells. Seemingly opposite conclusions are obtained, which, however, may be caused by randomly acquired differences rather than being related to the GOI. (D) Interaction diagram using the average values of panels B and C. By averaging the results of independently modified cell lines, random differences can be expected to cancel out and the only remaining systematic difference is the GOI, which is now seen to have no effect.

Although situations as just exemplified permit the interpretation that the GOI endows our cells with the observed phenotype, it should be emphasized that doxycycline can still interfere with the experimental outcome. First, doxycycline can directly attenuate or enhance the phenotype. In the case of increased proliferation mentioned in the preceding paragraph, the GOI can be expected to act even more strongly, as doxycycline likely counteracted its effect. Second, the observed phenotype might be an indirect outcome due to synergistic or antagonistic interactions between the GOI and doxycycline. A GOI might, for instance, not affect proliferation directly but may cause the cells to become more sensitive to doxycycline, which in turn causes them to proliferate slower. In such a case, attributing this effect to the GOI would be inaccurate.

## GENERATING ADDITIONAL CONTROL CELL LINES

Besides omitting doxycycline from the culture, a second commonly used control strategy is the use of control cells that do not overexpress the GOI upon administration of doxycycline. One option is genetically nonmodified cells (i.e., wild-type or WT cells). In the test case executed in our lab, this led to the conclusion that the GOI increased the observed cell number ("WT + Doxy" and "iGOI + Doxy"; Figure 1A), which was opposite to the previous interpretation. Again, however, a major flaw in the design—this time related to the "batch effects" mentioned before—hampered the correct interpretation of the results.

Recall that the iGOI cells were extensively manipulated by integration of the transgene and subsequent antibiotic selection, while WT cells were not exposed to any of these experimental procedures. These procedures, however, can drastically alter the (epi)genetic characteristics (and consequently the behavior) of the selected cells compared with the parental cells (Halliwell et al., 2020). First of all, genome editing with engineered nucleases or nickases (e.g., TALENs, zinc-fingers, CRISPR-Cas9) might induce both on- and off-target damage. Although some studies have reported these events to be rare in PSCs (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014), engineered cells should always undergo some sort of quality control either by classical karyotyping or, preferably, by more sensitive methods such as array-CGH or—if affordable—whole genome sequencing. Second, due to the heterogeneous constitution of the parental cell population (often referred to as clonal heterogeneity [Suzuki et al., 2014; Veres et al., 2014; Halliwell et al., 2020]), the antibiotic-selective pressure will likely favor the survival of a certain subpopulation possessing a selective growth advantage. Of note, the raised concerns also apply to cancer cells (Ben-David et al., 2019), where they can be expected to have even bigger implications as it is well-known that these cells have a higher spontaneous mutation rate than PSCs (Andrews, 2021) and are more prone to off-target effects of gene editing (Yee, 2016).

When we compared the proliferation rates of WT and iGOI cells in the absence of doxycycline (i.e., "WT – Doxy" and "iGOI – Doxy"), we observed that the iGOI cells proliferated faster than the WT cells, suggesting that the inherent properties of the cells were altered by the genome editing procedure (Figure 1A). Any observed difference between "WT + Doxy" and "iGOI + Doxy" could thus have been unrelated to the overexpression of the GOI but instead be a direct consequence of modifying the cells.

A possible strategy to solve this problem would be the replacement of WT cells by a control group that is more similar to the iGOI cells. This can be done by exposing a batch of control cells (i.e., iCTRL cells) to a comparable experimental procedure as the iGOI cells, that is, transgene delivery and subsequent selection. Such an approach naturally raises the question of which transgene should be used as the control. Without doubt, the best option would be the exact same vector that was used for generating the iGOI cells, with the GOI being replaced by a mutated, nonfunctional form. In this way, both cell types need similar cellular resources to express the transgene and similar side effects of the overexpressed gene can be expected. Unfortunately, such mutations remain unknown for many genes. Therefore, one often needs to settle for the second-best option, be it an empty vector or a different—supposedly inert—protein such as green fluorescent protein (GFP). Overexpressing GFP has the advantage that the cells need to devote resources to transcription and translation of the protein like the iGOI cells do. On the other hand, evidence exists that GFP might affect cell survival and behavior (Ansari et al., 2016; Ganini et al., 2017). An empty vector, expressing only the antibiotic resistance, does not suffer from this last issue, but neither do the cells need to expend the same cellular resources for transcription and translation (only the resistance gene is overexpressed, rather than the GOI and the resistance gene). Which of these two approaches is to be preferred often depends on the research question under investigation, and no universally best choice exists.

Undoubtedly, any of the procedural controls just described would resemble the iGOI cells to a better extent than the WT cells did. Nevertheless, the chances for them being identical are rather small: because of independent generation of the iGOI and iCTRL cell batches, they may have acquired different (epi)genetic changes—whose functional implications might be unknown or remain undetected—as the result of gene editing and subsequent selection. Accordingly, systematic phenotypic differences between the two treatment groups may be caused by these distinct (epi)genetic changes, rather than being related to the GOI. Indeed, from Figure 1A it is clear that in the absence of doxycycline, iCTRL cells grow slightly faster than iGOI cells and thus behave differently. So strictly speaking, one can still not distinguish an effect of the GOI from potential batch effects.

## TESTING FOR INTERACTION EFFECTS

In an attempt to prevent misinterpretation due to potential batch effects, one can account for any differences between the "iCTRL – Doxy" and "iGOI – Doxy" cells (which would reflect how those batch effects influence the phenotype of interest) when analyzing the changes in the "+Doxy" condition. In other words, one can test whether the iGOI cells react differently to doxycycline than the iCTRL cells do. The correct statistical means for doing this is to perform a significance test for so-called *interaction effects*, rather than simply comparing the "iGOI + Doxy" condition with "iCTRL + Doxy" and the "iGOI – Doxy" condition with "iCTRL – Doxy" by separate significance tests, while hoping that the former is significant and the latter not. Although, admittedly, both approaches might often lead to the same conclusions, they are generally not the same, and using the wrong test can lead to inaccurate conclusions (Keppel, 1991).

Once a significant interaction has been detected, the results can be interpreted as shown in the interaction diagram in Figure 1B: assuming that any difference in the control group can be entirely ascribed to doxycycline and that doxycycline affects the proliferation of both cell types in an identical manner, any additional change in the iGOI cells can be attributed to the GOI. The latter assumption, however, is by no means self-evident under the considered circumstances. Just as the basal proliferation rate of the iCTRL cells is higher than in iGOI cells, the latter can—just by chance—be more sensitive to doxycycline, causing the experimental outcome. Accordingly, any observed phenotype can be ascribed to the GOI only
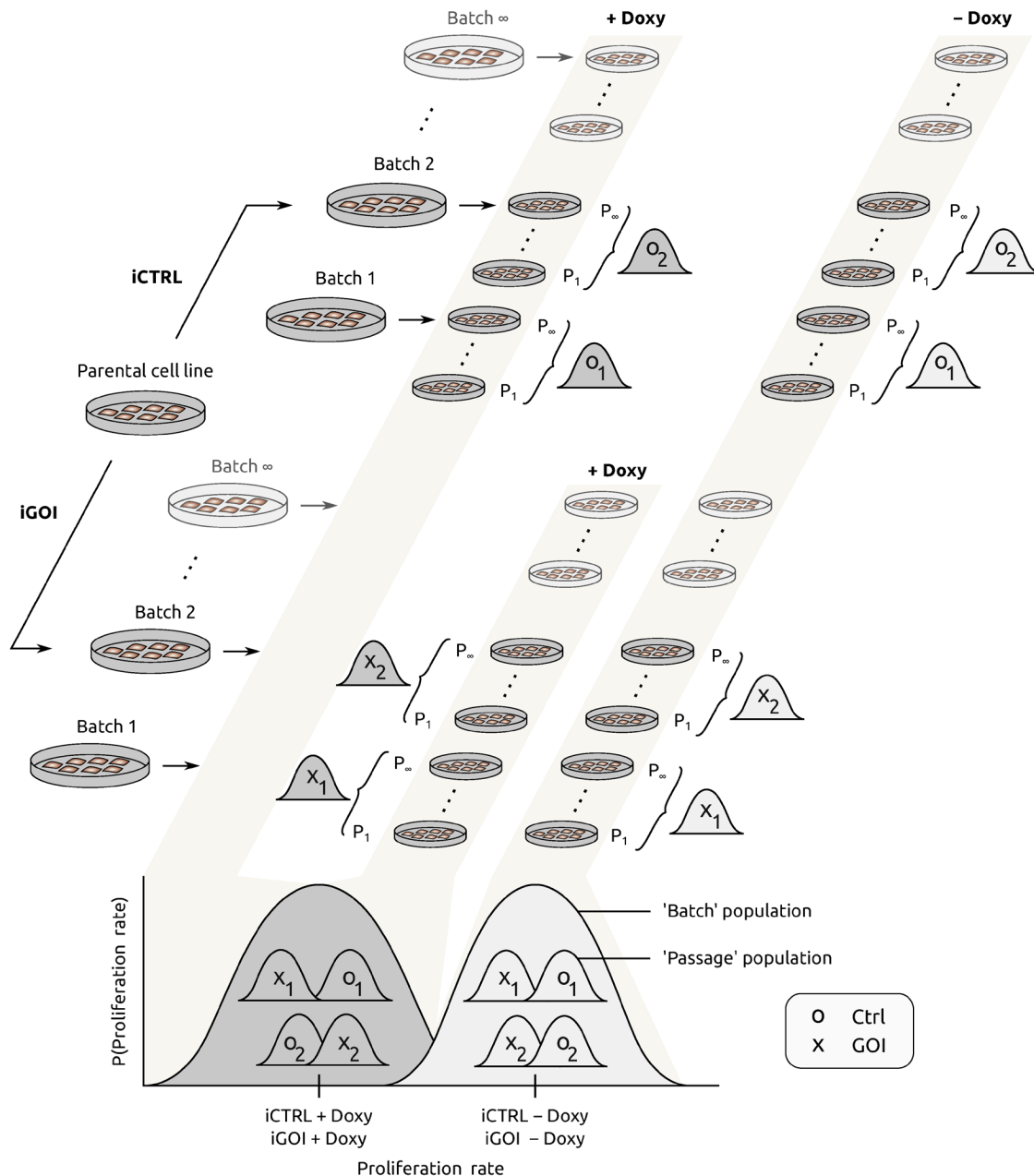
**FIGURE 2:** Replicating at the correct level. In Figure 1, B and C, we sampled five replicate passages from a population consisting of all possible passages ($P_1,\ldots,P_\infty$) of a single modified iGOI or iCTRL cell batch. These "passage" populations are here shown as the small bell-shaped distributions (Figure 1B being "batch 1" and Figure 1C being "batch 2"). Alternatively, one can consider each independently modified cell batch as a replicate sample from a population of all possible iGOI or iCTRL batches (i.e., the big bell-shaped distributions, referred to as "batch" populations). This is similar to Figure 1D, where for each of the iGOI and iCTRL groups, two independently modified cell batches were used as replicates. Note how in our example, the "iGOI + Doxy" and "iCTRL + Doxy" batches belong to the same (dark gray) "batch" population, as the GOI has no effect. This, however, is not generally the case: if the GOI would reduce or increase proliferation, the "iGOI + Doxy" population would be shifted to the left or right, respectively. In the absence of doxycycline on the other hand, all possible batches of both iGOI and iCTRL cells do—per definition—belong to the same (light gray) "batch" population (see main text for details). As replicate passages from the same cell batch are arguably more alike than independently modified cell batches, the distribution of the "passage" population has a smaller variance (i.e., is narrower) than the "batch" population. Therefore, one will readily find significant differences between "passage" populations (e.g., $x_1$ vs. $o_1$ and $x_2$ vs. $o_2$). Such a potential difference may, however, be the consequence of randomly acquired (epi)genetic differences, rather than being related to the GOI. If on the other hand, one independently generates multiple batches of genetically modified cells, and thus compares the "batch" populations, such random differences are more likely to average out, resulting in more reliable conclusions.

if one is willing to make unverifiable assumptions. Next, we will discuss a possible strategy to overcome this difficulty.

## REPLICATING AT THE CORRECT LEVEL

Suppose for a moment that the experiment in Figure 1B is to be repeated, that is, we independently generate a new batch of iGOI and iCTRL cells and again use five passages of these newly generated cells as replicates (Figure 1C). Repeating the previously described analysis based on interaction effects now leads to the opposite conclusion that the GOI increases the proliferation rate. Again, however, this outcome can simply be caused by random differences in doxycycline sensitivity between iCTRL and iGOI cells.

Having these two—seemingly contradictory—experiments, a correct interpretation lies in averaging their outcome (Figure 1D). By doing so, random differences can be expected to average out and the only systematic difference between the treatment groups remains to be the overexpression of the GOI, as is desired to correctly interpret the results. Generalizing this reasoning provides us with a new strategy to perform the experiment: multiple, independently generated iGOI and iCTRL cell lines should serve as replicates (referred to as "batch replication"), rather than different passages of a single modified iGOI and iCTRL batch (as we have done so far, further referred to as "passage replication"). This newly introduced "batch replication" has several important implications:

*1. Justifying the assumption of equal doxycycline sensitivity.* As alluded to above (Figure 1D), the idea behind independently modifying several batches of iCTRL and iGOI cells is the fact that randomly acquired (epi)genomic and/or phenotypic differences will hopefully average out. Consequently, we can more safely assume that cells in distinct treatment groups are equally sensitive to doxycycline and the observed phenotype can be ascribed to the GOI whenever a significant interaction effect is detected (following an analysis similar to that in Figure 1B).

Unfortunately, one can never really assure whether all random differences indeed canceled out. Lack of any significant phenotypic differences between the "iCTRL – Doxy" and "iGOI – Doxy" groups, however, can serve as a necessary condition—although not a sufficient one—for this assumption to hold true (this explains why, in the absence of doxycycline, all possible batches of both iCTRL and iGOI cells belong to the same population in Figure 2). Indeed, it would make little sense to argue that random differences between iCTRL and iGOI cells averaged out if such differences still exist in the "–Doxy" treatment group.

*2. Broadening the scope of inferences.* Using "batch replication" implies that the statistical populations that we compare will differ from the populations we compare when using "passage replication." Indeed, the latter populations have a much narrower distribution than the former ones (Figure 2), that is, "replicate passages" are more similar than "replicate batches."[1] Although this larger variability between multiple batches makes it harder to find significant differences (as the sensitivity of a test is typically inversely correlated with the variance), it does positively affect the scope of our inferences. Specifically, our findings now apply to the parental cell line used, whereas inferences based on replicate passages applied only to that single modified batch. Add to this the fact that the interpre-

tations based on replicate passages are more likely to be inaccurate (i.e., they are, as extensively discussed above, more likely to be the effect of randomly acquired (epi)genetic differences rather than being related to the GOI), and it becomes clear that generating multiple batches of independently genetically modified cells is the preferred strategy to arrive at scientifically sound conclusions.

## CONCLUSIONS

Here, we put forth two reasons why the use of doxycycline inducible Tet-On systems is not as straightforward as it might first seem:

1. The experimental outcome might be confounded with side effects of doxycycline when comparing "+Doxy" and "–Doxy" conditions. Of note, similar concerns can be raised for other inducible expression systems, such as Tet-Off, CreERT2-LoxP (Feil *et al.*, 1997), and Cumate (Mullick *et al.*, 2006), whenever the readout is performed while the inducing compound is present in the system, or shortly after it was removed. [2]As recently discussed elsewhere, such adverse effects also should be considered when performing in vivo animal experiments (Wüst *et al.*, 2020).

2. The experimental outcome might be confounded by batch specific (epi)genetic alterations when comparing multiple, independently modified cell lines. Importantly, such batch effects are not caused by the inducible system per se. Indeed, even under standard culture conditions, genomic evolution is well-known to induce discrepancies between cell populations and can jeopardize reproducibility in research (Ben-David *et al.*, 2019). Accordingly, batch effects should be considered any time cells are exposed to a selective pressure (e.g., viral transduction or antibiotic selection), even in the case of constitutive overexpression.

It should be emphasized that circumventing these pitfalls by simply abandoning the use of doxycycline is probably not the best option, as they also apply to most of the alternatives. Furthermore, doxycycline inducible systems certainly have their merits, most notably that they represent reversible models. Therefore, a better strategy could be what we called "batch replication." Admittedly, this approach is more expensive and time consuming and might therefore not always be practically feasible. Fortunately, simply comparing "+Doxy" and "–Doxy" conditions or using a single modified cell batch per treatment group (and using "passage replication"), does not necessarily invalidate experimental conclusions in practice. One should, however, be aware of the lurking risk of confounding and realize that interpretation of such experiments relies on certain assumptions. Identifying these assumptions and potential confounding factors, and preferably explicitly reporting them in publications, is paramount to minimize the risk for misinterpretation of—either one's own or published—experimental results.

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[1]It should be mentioned that his argument is purely based on intuition here and not on actual data, as data comparing the different sources of variability in (stem) cell culture experiments are—unfortunately—rarely available.

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[2]One advantage of the CreERT2-LoxP system over, for instance, the Tet-On/Off system, is the fact that one can add tamoxifen only transiently while inducing a permanent overexpression. As such, the readout of the experiment can be collected long after tamoxifen is removed and the risk for confounding can be minimized. Unfortunately, the CreERT2-LoxP system does not allow reversible overexpression, if desired.

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