

INVITED REVIEW

GFP Transgenic Animals in Biomedical Research: A Review of Potential Disadvantages

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Summary

Green Fluorescent protein (GFP) transgenic animals are accepted tools for studying various physiological processes, including organ development and cell migration. However, several *in vivo* studies claimed that GFP may impair transgenic animals' health. Glomerulosclerosis was observed in transgenic mice and rabbits with ubiquitous reporter protein expression. Heart-specific GFP expression evoked dilated cardiomyopathy and altered cardiac function in transgenic mouse and zebrafish lines, respectively. Moreover, growth retardation and increased axon swelling were observed in GFP and yellow fluorescent protein (YFP) transgenic mice, respectively. This review will focus on the potential drawbacks of the applications of GFP transgenic animals in biomedical research.

Key words

GFP • Transgenic animals • Glomerulosclerosis • Dilated cardiomyopathy

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GFP as a fluorescent marker in transgenic animals

GFP, which was originally discovered in jellyfish *Aequorea victoria*, absorbs blue light and emits green fluorescence without any exogenous substrates (Shimomura *et al.* 1962). Several variants of GFP with modified fluorescence spectra were designed in the last two decades, including enhanced GFP (EGFP) (Cormack

et al. 1996), YFP (Wachter *et al.* 1998), Venus (Nagai *et al.* 2002) etc. EGFP had increased extinction and more efficient folding, whilst Venus showed decreased sensitivity to both acidosis and Cl⁻ compared to wild-type GFP, for more details, see review (Zimmer 2009). The development of the expression vector composed of the chicken β -actin promoter and cytomegalovirus enhancer (CAGGS), offered a chance for the researchers to create transgenic animals with ubiquitous reporter protein expression (Niwa *et al.* 1991). After this innovation, GFP or its derivatives were successfully integrated into the genome of laboratory and livestock animals, e.g. mouse (Okabe *et al.* 1997), pig (Garrels *et al.* 2011, Park *et al.* 2001), rabbit (Katter *et al.* 2013, Takahashi *et al.* 2007), etc. Recently, EGFP transgenic animals have been used for modelling human diseases (Garcia Diaz *et al.* 2016, Shetty *et al.* 2019), tissue engineering (Hirakata *et al.* 2016) and helped the researchers to reveal the platelet biogenesis in lungs (Lefrancais *et al.* 2017). Moreover, utilizing of GFP infected chondrocytes was valuable for studying developmental dysplasia and dislocation of the hip in rats (Ning *et al.* 2019). Initially, most of the studies suggested that GFP is biologically inert. After the creation of GFP transgenic mice, the non-toxic nature of GFP in living cells was questioned by certain researchers (Liu *et al.* 1999).

Immunogenicity of GFP

Intravenous administration of wild type pre-leukemia cells cause mortality in Balb/c mice while

EGFP transformed leukemia cells did not cause systemic leukemia (Stripecke *et al.* 1999). Further studies indicated that EGFP is highly immunogenic in Balb/c but only moderately immunogenic in other strains like C57BL/6. Not only the strain but also the route of administration of cells influence the level of immune response against EGFP expressing cells (Skelton *et al.* 2001).

GFP-evoked dilated cardiomyopathy and altered cardiac function

Dilated cardiomyopathy was detected in transgenic mice with heart-specific GFP overexpression (Huang *et al.* 2000). In four independent transgenic mouse lines, cardiac-specific GFP expression was driven by the α -myosin heavy chain gene (α -MyHC) promoter. Significantly increased heart mass/body mass ratio, four-chamber dilation, relatively thin myocardium was observed in two out of four transgenic FVB/N strains compared to non-transgenic controls. More than half of the 5-weeks old male mice died due to congestive heart failure from one of the two transgenic lines, while mice from the other line developed systolic dysfunction at 4 months of age. The severity of dilated cardiomyopathy

positively correlated to the GFP expression level in the heart, suggesting a dose-dependent adverse effect of GFP (Huang *et al.* 2000). Age-dependent abnormalities in cardiac morphology and function were published using transgenic zebrafish lines with heart-specific GFP expression. Decreased heart rate, stroke volume, and cardiac output (Ho *et al.* 2007) and altered cardiac function (Avey *et al.* 2018) were measured in 8-days old and adult myosin light chain 2 gene (*cm1c2*)-GFP transgenic zebrafishes, respectively, but GFP exerts no detrimental actions on 3-days old zebrafish hearts (Huang *et al.* 2011). Several attempts were made to discover how GFP overexpression affects cardiac physiology and induces dilated cardiomyopathy. In vitro studies reported that EGFP expression impairs actin-myosin interactions and contractile function of muscle cells (Agbulut *et al.* 2006, Agbulut *et al.* 2007), but other research groups did not confirm these findings (Resnicow *et al.* 2008). In another study, heart-specific co-expression of EGFP and a calmodulin II kinase (CaMKII) inhibitory peptide, auto-camtide-3-inhibitor successfully counteracted the EGFP-evoked left ventricular dilatation and dysfunction, thus, EGFP may cause increased CaMKII activity in cardiomyocytes which led to dilated cardiomyopathy (Khoo *et al.* 2008), details are in Table 1. Nevertheless,

Table 1. The collection of the *in vivo* data of GFP-induced pathology in transgenic animals

Affected organ systems	Transgenic strains	Symptoms	Possible explanations
Cardiovascular system	α -MyHC-EGFP mice	Dilated cardiomyopathy, premature death (Huang <i>et al.</i> 2000).	Impaired actin-myosin interactions (Agbulut <i>et al.</i> 2006), increased CaMKII activity (Khoo <i>et al.</i> 2008).
	<i>cm1c2</i> -EGFP zebrafish	Altered cardiac function (Avey <i>et al.</i> 2018; Ho <i>et al.</i> 2007; Huang <i>et al.</i> 2011).	
Urinary tract	CAGGS-EGFP mice	Glomerulosclerosis, proteinuria, tubulo-interstitial injury (Guo <i>et al.</i> 2007).	Defective polyubiquitination (Baens <i>et al.</i> 2006), oxidative stress (Ganini <i>et al.</i> 2017).
	GABARAP-EGFP mice CAGGS-Venus rabbits	Glomerulosclerosis, proteinuria (Takagi-Akiba <i>et al.</i> 2012), (Liptak <i>et al.</i> 2018).	
Central nervous system	lacZ-GFP mice	Growth retardation, premature death (Krestel <i>et al.</i> 2004).	GFP/YFP excessive aggregation/accumulation.
	Thy-1 YFP mice	Increased axon swelling (Bridge <i>et al.</i> 2009).	

Abbreviations: α -MyHC: α -myosin heavy chain gene, *cm1c2*: myosin light chain 2 gene, CaMKII: calmodulin II kinase, GABARAP: γ -Aminobutyric acid A receptor-associated protein.

mild hypertrophy and cardiomyopathy was observed in α -MyHC tetracycline transactivator transgenic mice (McCloskey *et al.* 2005), indicating that overexpression of any protein in the heart under the control of α -MyHC promoter may cause cardiomyopathy.

GFP-evoked glomerulosclerosis

In most cases, ubiquitous expression EGFP or its variants under the control of the CAGGS promoter did not affect fecundity (Garrels *et al.* 2012, Hoffmann *et al.* 2016), life span and had no measurable detrimental effect (Chou *et al.* 2014, Garrels *et al.* 2011, Yum *et al.* 2018).

Nowadays, CAGGS-EGFP transgenic mice (C57BL/6-Tg(CAG-EGFP)10sb/J, Jackson Laboratories) with ubiquitous reporter protein expression (Okabe *et al.* 1997) are one of the most popular transgenic animals in biomedical research. However, 5-weeks old CAGGS-EGFP transgenic mice developed mild glomerulosclerosis and proteinuria (Guo *et al.* 2007). The pathological progression was slow, age-dependent and did not affect the life-span of the mice. Only CAGGS-EGFP transgenic mice with the highest EGFP expression in their glomeruli developed glomerulosclerosis. Three other transgenic strains with lower glomerular GFP expression showed normal renal histology, suggesting that high EGFP levels may have harmful effects on the glomeruli (Guo *et al.* 2007). Supporting these findings, more severe proteinuria and glomerulosclerosis were observed in an EGFP- γ -Aminobutyric acid A receptor-associated protein (GABARAP) transgenic mouse strain after a single doxorubicin injection compared with non-transgenic mice (Takagi-Akiba *et al.* 2012). Mild proteinuria, focal segmental glomerulosclerosis (FSGS) and glomerulomegaly were also detected in heterozygote CAGGS-Venus transgenic rabbits, while homozygote Venus transgenic rabbits developed FSGS, glomerulomegaly and microscopic hematuria, but not proteinuria (Liptak *et al.* 2018). Similarly to CAGGS-EGFP transgenic mice, the mild glomerulosclerosis did not affect the life span. The Venus transgene was integrated at chromosome 8, at 61471914 bp (Katter *et al.* 2013) in the intron 12–13 of the diaphanous related formin 3 gene (DIAPH3, Ensembl gene ID: ENSOCUG00000001719), thus, it is unlikely that the integration site of the transgene caused the FSGS. These data suggested that glomerulosclerosis in transgenic animals overexpressing the reporter GFP or its variants (EGFP, Venus) is not limited to mice. The underlying

mechanisms of GFP-associated glomerulosclerosis are not clear, however, defective polyubiquitination (Baens *et al.* 2006) and oxidative stress (Ganini *et al.* 2017, Goto *et al.* 2003) may have a role in the development of glomerular lesions. EGFP produces H_2O_2 and superoxide anion during its maturation in the presence of NADH, but these findings were restricted to *E. coli* and HeLA cell lines to date (Ganini *et al.* 2017) (Table 1).

GFP-induced neuropathology

EGFP and β -galactosidase co-expression in the forebrain caused growth retardation, weakness and premature death in 25–30 days old transgenic mice from two independent lines (Krestel *et al.* 2004). GFP alone did not have any of the abovementioned neuropathological effects in a third transgenic mouse line. The cytoplasmic aggregation of EGFP and β -galactosidase could be responsible for the premature death in the two transgenic lines. Doxycycline and granulocyte colony-stimulating factor (GCSF) treatment were effective in the prevention of early death of transgenic mice co-expressing EGFP and β -galactosidase, but did not prevent them from growth retardation (Krestel *et al.* 2004). Doxycycline exerted its effect *via* downregulation of the EGFP expression, while GCSF had neuroprotective effect which was published earlier (Schabitz *et al.* 2003).

In YFP transgenic mice, more severe axon swelling was observed in gracile tract, gracile nucleus and dorsal roots compared to non-transgenic mice at 8 and 12 months of age (Bridge *et al.* 2009). YFP expression was driven by the neuron-specific Thy-1 promoter in the transgenic mice. Unfortunately, the exact genomic position of the YFP coding transgene is not available. Increased axon swelling was attributed by the excessive accumulation of the YFP in the central nervous system and not the position effect of the transgene (Bridge *et al.* 2009). These observations were underlined by an independent research group later (Gatto *et al.* 2015) (Table 1).

Conclusions

In the majority of GFP transgenic animal lines, GFP did not have any detectable harmful effect on animals' health, but there were a few exceptions. Heart-specific GFP overexpression caused dilated cardiomyopathy in mice and altered cardiac function in

zebrafish. Transgenic mouse and rabbit strains with CAGGS promoter-driven ubiquitous EGFP or Venus expression developed mild glomerulosclerosis and proteinuria. Neuron-specific expression of YFP and co-expression of EGFP and β -galactosidase caused pathologic symptoms in mouse strains created by different research groups. These findings should be considered when *in vivo* studies using GFP transgenic animals are designed.

Conflict of Interest

There is no conflict of interest.

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