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The Presence of GFP in Embryos and its Effect on Cells

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Abstract

GFP is a 238 amino acid ~27 kDa protein derived from the crystal jellyfish Aequorea Victoria. was used to produce transgenic chickens, transgene expression was primarily in the pancreas and to a lesser extent in the liver, skin, muscles, and intestinal lining. The study describes the expression of GFP in various tissues from fetal, neonatal and adult animals. GFP expression in brain, lung, liver, and islet tissues was limited early in development, but was consistently strong in exocrine pancreas, kidney, cardiac, and skeletal muscle. The CAG promoter, which was hypothesized to induce ubiquitous protein expression, may be responsible for the differences in expression.

Keywords: GFP; Embryo; Cell; Amino acid; Transgenic; Protein

Presence of GFP in chick embryos

The embryonic chick is a valuable model organism used to study the development of vertebrates. In addition to their availability and the low cost of fertilized eggs, early stages of embryonic chicks are readily available at Ovo for imaging and experimental manipulation. During the first 3 days of incubation, the chick embryo, starting as a blastodisc, undergoes gastrulation, neurulation, and several morphogenetic movements, forming various cranial structures. During this developmental period, embryos can be easily accessed and manipulated at different developmental times by “windowing” the eggshell and then re-incubated to the desired late embryonic stages. Using this approach, numerous methods such as DII injections [1] and tissue transplantation [2,3] or tissue ablation have been used to study cell fate and pattern formation. In addition, gain and loss assays, including electroporation of DNA, RNAi, morpholines, and viral constructs, are now widely used in molecular studies in chick embryos [4]. It was found that GFP gene expression in blastoderms was observed after 24 hours of cultivation under a fluorescent stereomicroscope (SZX 12, Olympus, Tokyo, Japan), and the true development of embryos with many nuclei, [5], blastoderms were stained with 4,6-diamidino-2- phenylindole (DAPI), DNA-specific dye, generally as described in [6]. Despite their versatility as model organisms for the study of developmental biology, chicken embryos are rapidly surrounded by extraembryonic membranes, making experimental manipulations difficult on the last embryonic day (E5). The chick embryo is initially covered with a transparent yolk membrane that separates it from the albumen. Starting at stage HH 12 [7], the amnion and chorion membranes overlap the hindbrain and tail bud, grow towards each other, and fuse to cover the entire embryo at stage 18. Around the same time, another membrane, the allantois, forms as a balloon-like structure in the region of the posterior intestine of the embryo. The allantois fuses with the chorium to form the chorioallantoic membrane, which stores nitrogen waste and is involved in respiration and calcium transport. The chorioallantoin membrane grows rapidly and covers most of the embryo at the 20th stage (E5). During early development,

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embryos can be easily accessed through the tunica lutea or chorion, as well as by transection of the amniotic membrane. However, access to an E5 or earlier embryo through an overlaid chorioallantoic membrane is lethal. Therefore, most developmental biology studies using chick embryos in ovo are limited to relatively early developmental stages prior to critical periods of organogenesis that may require different signals and cellular interactions. Several genes associated with cellular interactions and differentiation during organogenesis of the eye, ear, brain, skin, and tissues such as bone and cartilage are either transiently expressed or initiate expression at later stages of embryogenesis. To increase availability to later stages (older than E4), methods such as ex ovo culture of embryonic chicks have been developed [8,9]. While these techniques overcome the complications resulting from embryo adherence to the eggshell and increased access to the embryo, the chorioallantoic membranes expand and obscure the embryo from manipulation in the later stages. In addition, ex ovo grown chick embryos exhibit severe growth retardation, likely due to a Ca2+ deficiency naturally provided by the eggshell [10]. So far, no attempt has been made to remove the barriers to handling late-stage chick embryos in ovo. Current studies of organogenesis can only be carried out *in vitro* on tissue that is isolated from the influence of the endogenous embryonic environment, which can influence gene expression and key cellular interactions. In this study, we report a new method that further strengthens the chick embryo as a developmental biology model. This stepwise method combines the preparation of fertilized hen eggs at different developmental times with careful dissection and movement of extra-embryonic membranes to increase the availability of late-stage embryos. Embryos exposed to this technique are viable and readily available in the ovo for tissue manipulation during organogenesis. We show that different areas of exposed E7 embryos are readily accessible for manipulations such as injection with RCAS-GFP expressing cells. After two days of re-incubation, the injected cells can be monitored by monitoring GFP expression in various tissues. Late-stage embryos subjected to this technique can be manipulated using standard approaches to study gene function, cell interactions, tissue regeneration, and stem cell potential during organogenesis [11]. In another study, the heart developed after 50 h of incubation, the virus solution was injected into the heart of the embryos to effectively deliver the vector to the entire embryo, including primary germ cells, using the circulatory system. Maximum expression was observed when viral vectors were injected into embryos after 55 hours of incubation, stage 14-16 in the setting of Hamburger and Hami [7]. High-level expression of the transgene was also observed in the gonads. This suggested that the transgene could be passed on to offspring without gene silencing [12]. EGFP has been found in trophoblasts from the earliest stage of implantation, studied at embryonic day 7.5. During embryonic development, EGFP has shed a large number of c-fms-positive macrophages, including those derived from the yolk sac. In adult mice In the EGFP study, the location matched known FP/80-positive macrophage populations, including skin Langerhans cells, and allowed for convenient sorting of isolated tissue macrophages from disaggregated tissue. EGFP expression in transgenic mice was dependent on intron 2, since no lines with detectable EGFP expression were obtained where the entire intron 2 or the conserved FIRE enhancer element (intact Fms regulatory element) was removed. Therefore, we have identified the elements required for the generation of myeloid and trophoblastic transgenes, as well as a model system for studying the mononuclear phagocyte [13]. This has been found in some studies. The results showed that 48% of embryonic cells injected after 55 hours of incubation had, on average, the vector sequence, while about 1% of embryonic cells were infected with blastoderm after injection. The expression level per copy number of the transgene for blastodermal injection is 40 times lower than for injection after 55 h of incubation [12,14]. The viral titer was found to significantly affect the expression of β-galactosidase. Since expression was weak with a viral titer below 107 IU/mL, at least 108 IU/mL is required for high level expression of the transgene, although injection of more than 5 x 109 IU/mL resulted in severe toxic effects on embryonic development (data not shown). From these results, it is preferable to inject viral vectors with a titer greater than 108 IU/ml into the heart of embryos after 55 h of incubation to obtain the highest level of transgene expression [15,16]. The chick embryo provides an excellent model system for studying gene function and regulation during embryonic development *in vivo*. The Ovo electroporation technique has been recognized as a powerful technique for effectively inducing exogenous genes in the chick embryo [17]. Through electrophoresis, one or more cDNA plasmids, interference RNAs, or morpholines can be introduced into embryonic cells to overexpress genes of interest or reset their expression in limited areas [18]. Although the transfected cDNA plasmid is not incorporated into the host's chromosomal DNA, expression of exogenous genes may continue for more than 8–11 days after electroporation [10]. Because the chick embryo can be easily accessed and manipulated, electroporation has been widely used in ovo to investigate questions of vertebrate development [19]. The method has been applied to studies of neurogenesis and neuronal differentiation [20], axon outgrowth [21], somitogenesis [22], limb development [23], skeletal muscle function [24], eye development [25], and gene therapy [26]. In general, in ovo, electroporation is only applied to chick embryos in the early stages of incubation (i.e., embryos younger than stage 20 by [7,18]. In later stages (stage 22 and older), several experimental problems in ovo electroporation occur, making electroporation extremely difficult to perform. As a solution to these problems, we used ex ovo (unenveloped) culture for

electroporation in chick embryos [10]. the entire contents of the egg are transferred to the Petri dish system [8]. For example, to study the effect of functionally blocking antibodies [27], calcium deficient hypertension [28], atherosclerosis (eg, 50), muscle maturation [29], intravenous tracers [30], and testing of biosensors [31]. In the present work, we have demonstrated that genes (cadherin7 [Cad7] and green fluorescent protein [GFP]) can be successfully transferred to various regions of the central nervous system and muscles of chick embryos within 4-7 days of incubation (embryonic day E4 - E7; step 23-30). Our results indicate that the ex ovo electroporation system can be used to study gene function at relatively late stages of chick embryonic development [10].

The Effect of GFP on Cells

Green fluorescent protein (GFP) is the most commonly used reporter protein for monitoring gene expression and protein localization in a variety of living and fixed cells, including not only prokaryotes but also eukaryotes such as yeast, mammals, plants, and fish. In general, GFP is considered non-toxic to cells, although there are some reported side effects of GFP. In addition, the details of the molecular mechanism regarding the side effects of GFP remain unclear. Here we show that Ku80, but not XRCC4, plays an important role in the mechanism of resistance to enhanced GFP (EGFP) cytotoxicity. EGFP inhibited both cell proliferation and colony formation and induced cell death in Ku80 deficient hamster cells, ie xrs-6 cells. In addition, Ku80 attenuates EGFP-induced cytotoxicity in xrs-6 cells. EGFP-induced cytotoxicity was not observed in NHR-nucleus hamster cells with NHRJ nucleic cell, i. XR-1 cells. In addition, EGFP markedly improved X-ray-induced cytotoxicity in xrs-6 cells. These results indicate that Ku80 plays a key role in a new NHEJ-independent defense mechanism against EGFP-induced cytotoxicity. Care should be taken when considering the potential impact of a stress response mechanism, namely a mechanism capable of a Ku80-dependent mechanism of EGFP-induced cytotoxicity, which is activated even when using EGFP-expressing cells in which Ku80 functions normally [32]. In another study, initiation of the apoptosis cascade was postulated as a possible mechanism for GFP toxicity and cell death. After transfection with various GFP plasmid vectors, mouse embryonic and fibroblastic baby hamster kidney cells lost their GFP signals and disappeared after 120 h [33]. In this experiment, various morphological (loss of structural integrity) and molecular changes (redistribution of phosphatidylserine, an indicator of the initiation of the signaling apoptosis cascade to the cell surface) were reported in accordance with GFP-induced apoptosis. Another valuable feature indicative of cellular apoptosis was the presence of CPP32 (Caspace-3, an apoptotic protein) after GFP signal fading [17,34]. CPP32, a member of the interleukin-1β-converting enzyme (ICE) family, plays an important role in programmed cell death. CPP32 has been shown to be highly expressed in cells initiating apoptosis. Conversely, inactivation of CPP32 drastically reduces apoptosis; therefore, measurement of CPP32 activity is a reliable tool for monitoring apoptosis [35]. Consistent with morphological changes suggestive of apoptosis, the level of CPP32 expression is increased in cells transfected with the GFP plasmid compared to cells transfected with the empty vector [33]. In addition to initiating the apoptotic cascade, GFP-induced reactive oxygen production is associated with cellular toxicity and eventual death in GFP-expressing cells. MHC class I (H2-Kd) has previously been described as a natural eGFP epitope initiating CTL activation [36]. However, it remains unclear how the immunogenicity of GFP via the MHC I pathway is related to the increased reactive oxygen species (ROS) observed in various experiments. One proposed mechanism is the exocytosis of granzyme B (GrB) by activated CTLs. Activated CTLs induce exocytosis of GHB, perforin, and GH via the death receptor, FAS/FASL. GrB, once exocytated, facilitates the release of mitochondrial ROS through direct cleavage of caspas-3 and nuclear lamin [37,38].

Increased sensitivity of GFP expressing cells to anticancer drugs such as etoposide has been associated with increased levels of ROS in the cells. This finding is supported by increased levels of p53-dependent glutathione, which acts as a cellular defense mechanism in situations of oxidative stress [39]. Due to oxidative stress, neuroblastoma cell lines lacking CD80 showed increased sensitivity to cytotoxic agents when transduced with GFP, eGFP and YFP [14]. CD80 is a protein on activated B cells and is required for the stimulation signal needed to activate T cells. Regardless of immunogenicity, both CD80-negative and CD80-transduced neuroblastoma cells have been shown to significantly improve sensitivity to cytotoxic anticancer agents when transduced with GFP, eGFP, and YFP [15,40]. In addition to the general cellular toxicity of GFP, there is some evidence of organ-specific cytotoxicity of GFP. Overexpression of GFP in the heart caused wild-type mouse strains to develop dilated cardiomyopathy [34,41]. In this model, GFP expression was associated with a significant increase in the weight ratio between heart, four-chamber dilatation, and thin myocardium, suggestive of dilated cardiomyopathy in young rats. The observation of more severe cardiomyopathy in cells with higher GFP expression suggests dose-dependent effects [41]. Impaired actin-myosin interactions due to GFP cytotoxicity have also been reported. eGFP transduction of myotubes using a lentiviral vector demonstrated attenuated binding to excitation and decreased contractile function of myotubes due to binding of GFP to the myosin-actin binding site [42,43]. Surprisingly [15,44], reactive gliosis and apoptosis of the forebrain region induced by neurodegeneration and cell death were confirmed in the co-expression of eGFP and β-galactosidase. Attachment of a

fluorescent protein to a protein of interest often has no identifiable effects on protein function, structure, and localization [45]. However, in some cases, it can disrupt the function of the protein, and the expression of this construct can adversely affect cellular function. Therefore, the nature of the expression system should be considered. A potentially large increase in the level of protein expression can have a major impact on the subcellular localization of the fusion protein and its cellular function. It is preferred that the cells express the smallest amount of fusion protein that can still be clearly displayed. Expression of a simple fluorescent protein in a cell can induce cytotoxic effects depending on which type of fluorescent protein is used. In the study, the widespread use of EGFP as a live cell reporter is based on the assumption that it does not affect (important) cellular functions. However, a number of side effects have been reported. First, an association was noted between GFP expression and apoptosis induction in a number of cell lines [17,33]. In endothelial cells, GFP administration with various gene transfer vectors selectively induced HSP70, resulting in upregulation of cyclooxygenase-2 (COX-2) expression, followed by prostaglandin E2 production [46]. Recently, EGFP has been shown to disrupt actin-myosin interaction in cardiac muscle cells [42], which may be related to the extended cardiomyopathy phenotype previously reported in GFP transgenic mice [41]. In addition, co-expression of EGFP and beta-galactosidase in transgenic mouse neurons induced neuropathology and premature death [44]. Until now, the mechanism by which GFP provokes these defects remains undetermined. EGFP has been described to block both Lys63- and Lys48-related polyubiquitination and thus affect NF-κB and JNK signaling and p53 homeostasis [47]. While attractive as an *in vivo* tracking tool in ASC repopulation assays, GFP has several disadvantages. One disadvantage of GFP is that it can cause cell death. Intense excitation of a protein *in vitro* for long periods of time can generate free radicals that are quite toxic to cells [14,33]. The failure to obtain constitutively expression of GFP cell strains may also be due to the effects of DNA methylation. In the presence of an irreversible methyltransferase inhibitor, C3A human hepatoblastoma cells transfected with GFP showed significantly greater retention of GFP expression and showed higher levels of GFP production [48]. As a result, GFP has been more successfully used as a viable marker for experiments with short time scales (hours), while attempts to establish long-term (monthly) GFP-expressing cell strains have been largely unsuccessful [33,49]. The reported efficiency of establishing stable, constitutively expressing cell lines is extremely low [15,50-52].

Conclusion

Embryos from eggs from genetically modified chickens on the 18th day of incubation had the lowest absolute values in terms of linear indicators of individual body parts. Genetic engineering manipulations affect the morphology of the internal organs of embryos. In embryos of 18 days of age, the weight of the heart is 7.6 and 5.7% more compared to their peers, the weight of the liver is less, respectively, by 13.0 and 19.3%, the weight of the stomach is less by 4.4 and 20.8%, respectively (P≥0.95).

Acknowledgement

None.

Conflict of Interest

None.

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