

NOBEL PRIZE

Does incorporation of gene for green fluorescent protein in BP6 fibrosarcoma tumor cells depress their intraperitoneal growth in rats?

(In Honour of Nobel Prize Laureates 2008 – Osamu Shimomura, Martin Chalfie, Roger Y. Tsien)

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Abstract: This manuscript was in honour of Nobel Prize in chemistry “for the discovery and development of the green fluorescent protein, GFP” to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien, simultaneously a brief information about experience with GFP in experimental tumorigenesis used this study is also presented. The experimental data have showed that BP6 cells incorporated with GFP gene have had smaller ability to induce both experimental intraperitoneal and subcutaneous tumor process. It was anticipated that incorporation of GFP gene might change physiological properties of cytoskeleton and worsen adhesive characteristics of tumor cells. It was also supposed that aftertime GFP will enable to monitor proliferation of cells not only within experimental work, but also in human medicine. GFP could help (supposedly) as reporter of proliferation, but also can serve as “target” for guide of tumorigenesis inhibiting substances. These ideas which are consequences of our experiments we append as congratulation to Nobel Prize in chemistry of the 2008 (*Fig. 2, Ref. 44*). Full Text (Free, PDF) www.bmj.sk.

Key words: GFP, BP6 tumor cell line, Nobel Prize in chemistry 2008, neurobiology, tumorigenesis.

Knowledge is an extremely complicated process, especially in the field of human endeavour since the last few decades. Understanding of living is a remarkable phenomenon that interests not only contemporary generation of excellent scientists and thinkers, but it will certainly interest the coming generations too. Every new view or taking a novel scientific approach is the representation of search of a new way with simultaneously essence in itself.

The constant endeavour taken to understand the basis of living has lead to great progress in the field of scientific research. Primal pleasure of defining the tissue as a suspension, or syncytium of similar cells even after 170 years has now shifted to science at the molecular level. Despite the fact that many are process specified, it has not been possible to have a deep insight and hardly will it be. A plunge into problems at molecular level could

be connected with a threat of elementary thinking. It is not that a molecule is alive, but an organism as an unit is living. At the same time properties of complex protein molecules are more belonged to life than defining by simple physics and chemistry.

Live organisms are composed of molecules. The changes noted in these molecules represent heritable differences between living organisms. Proteins (like amino acids) arise from transcription of genetic information and represent the essence of life. They participate in every manifestation in nature, present in any stage of development. They form parts of a cell and interact with many other cellular and non-cellular molecules. Importance of each protein molecule is highlighted as consequences of their deficiency or absence. For example deficiency of dopamine in left frontal lobe was confirmed during depression.

The study of protein in real state, such as basic structural components of cells, enzymes or information molecules is done usually using antibodies. Such studies are limited on fixative samples as living tissue antibody binding on protein results in changes of function and behaviour of this protein. A great progress in the study of dynamic protein processes was the discovery of gene for green fluorescent protein (GFP) for which in October 2008 the Nobel Prize in chemistry was awarded. **Osamu Shimomura**, first isolated GFP from jellyfish *Aequorea victoria*, **Martin Chalfie** showed the practical relevance of this protein, and **Roger Y. Tsien** contributed to understanding fluorescence mechanism of GFP and by various modifications extended the

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palette of colours. Primal position as “a green crayon” thus is replaced by prominence position in revolution of cell processes imaging techniques.

History of green fluorescent protein

Luminescence is usual in many species of marine invertebrates. Many Coelenterata (Cnidaria) emit green fluorescence following mechanical damage. *Aequorea victoria* (water jellyfish) lives along the west coast of the Pacific Ocean in USA. Average adult size is about 7–10 cm in diameter and it is shaped like a hemispherical umbrella. While during 60's to 80's *Aequorea* was occurring in high-density swarms spreading on the water surface during summer months, at present it has noticed a marked decrease in its incidence. *Aequorea* had interested many research groups for a long time already. The light organs consist of about 10 dozen tiny granules distributed along the edge of the umbrella making a full circle, each consisting of some 6000–70000 photocytes. In the cytoplasm of each cell are even finer granules containing the components required for mysterious night luminescence. Behind this physiological property is a hidden interesting mechanism, which uncovered the oldest of trio of Nobel Laureates, Osamu Shimomura. Cells of jellyfish release calcium that is binding on photoprotein aequorin and causing change in molecular structure. New form is unstable, represents a high level and therefore approaches immediate reaction associated with releasing Carbon Dioxide (CO₂), Calcium (Ca²⁺) and energy as light with wavelength 400 to 470 nm. This wavelength refer to blue colour of spectrum, but energy is absorbed by GFP and emits electromagnetic radiation with maximum in wavelength 505 nm, that we perceive as green colour.

GFP was discovered in 1962 by Japanese scientist Osamu Shimomura as a by-product of luminescent protein aequorin (1). This specialist in organic chemistry and marine biology during the first years of research (1955–1957) studied the bioluminescence and luciferin of ostracod Cypridina. The successful isolation and crystallization of luciferin (2) has enabled another investigation of this substance. In 1959 Shimomura received an invitation from Dr. Frank Johnson of Princeton University to study the bioluminescence of the jellyfish *Aequorea*. In the early summer of 1961 they travelled from Princeton to Friday Harbor (the State of Washington) because of highly incidence of the *Aequorea*, where he started with the study of emission mechanisms in animals and isolation of bioluminescent substances. The luminescent substance is extracted from the tissue under conditions that reversibly inhibit luminescence, or which cause a selective inactivation or removal of a co-factor necessary for light emission. During the purification of aequorin in *Aequorea* Shimomura found a protein with a bright green fluorescence and they called the protein “green protein” (3), later was more appropriately called “green fluorescent protein” (4). In 1974, Shimomura with team purified and crystallized GFP, measured its absorbance spectrum and fluorescence quantum yield, and also showed that aequorin could transfer its luminescence energy to GFP (5). Denatured GFP was proteolyzed, structural peptide was

analyzed, and was correctly proposed that the chromophore is a 4-(p-hydroxybenzylidene)imidazolidin-5-one attached to the peptide backbone through the 1- and 2-positions of the ring (6). When UV light or blue light hits the GFP chromophore that is excited. Then the chromophore gets rid of the energy and emits light, which is now in the green wavelength. The structure of GFP chromophore has been verified by Cody et al (1993) (7) and the X-ray structure of GFP was resolved in 1996 by two different groups (8, 9).

In 1987 Douglas Prasher came upon the idea to insert a gene for GFP to end of gene encoding another protein, before the stop codone. He sequenced gene for GFP and found out, that by his expression arises a small protein of 238 amino acids (10). Martin Chalfie heard about GFP for the first time in 1988 at a seminar about bioluminescent organisms. At this time he was dealing with the millimetre-long roundworm *Caenorhabditis elegans* and realised that this protein would be a fantastic tool for mapping the roundworm. He would like to connect the gene for GFP with various gene and by this way to watch activation of genes and expression of proteins. The first effort to heterological expression was realized in bacterial model of *Escherichia coli*. Many experts believed that a few different proteins of *Aequorea* were needed to produce the chromophore in GFP, but Chalfie's experiment showed that no other protein than GFP was needed. Protein exprimed in *v E. coli* has the same spectral properties as protein purified from *A. victoria*. Bacterial colony by this way genetically modified luminesces green light after light up by blue and UV light. In the next step, Chalfie placed the gene behind a promoter that is active in six touch receptor neurons in midget transparent roundworm *Caenorhabditis elegans*. In this case he used a new method when the GFP gene is not inserted on the end of gene, but on the beginning behind the promotor and this method is used predominantly up to this day. Modified gene for touch neuron they injected into genitals of adult (hermaphrodite) and observed that neurons of offsprings emit (11). This successful expression of GFP has established the basis of using GFP as a marker protein for gene expression, resulting in the present widespread popularity of GFP.

Tsien showed that this chemical reaction requires oxygen and explained how it can happen without the help of other proteins (12). By experimenting with the amino acid composition was able to develop new variants of GFP that shine more strongly and in quite different colours such as cyan, blue and yellow. The availability of GFP variants makes it possible to compare the dynamics of two distinct proteins simultaneously within cells.

BP6 cells with GFP in our experiments

In our laboratory, for study of complex interaction between tumor and organism, induction of tumour process by application of tumor cells BP6 is extensively used (13). This cell line opens up fair-sized successfulness within induction of tumor growth. However experiments that used application of tumor cell into animals are often limited by detection of tumor process in incipience. The real-time study and imaging of tumor cells in fresh

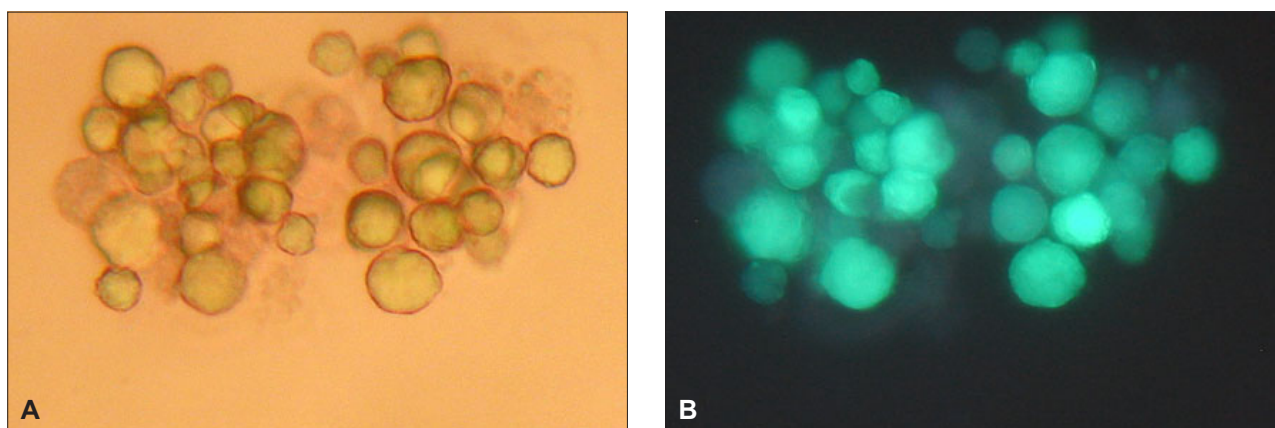


Fig. 1. (A) Fibrosarcoma BP6 cells transfected with GFP. (B) Imaging of fibrosarcoma BP6 cells transfected with GFP under the fluorescent microscope.

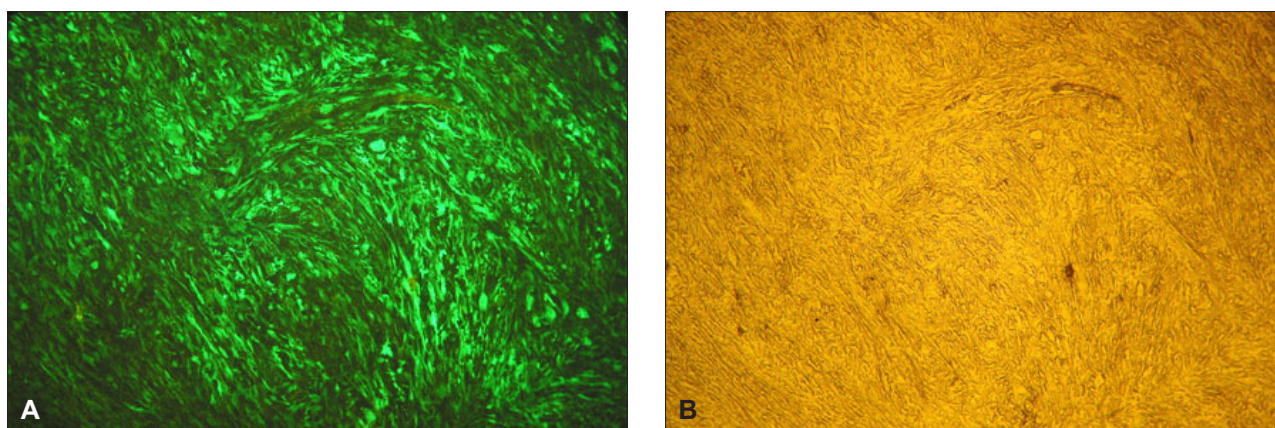


Fig. 2. (A) Fluorescence imaging of intraperitoneal BP6 GFP fibrosarcoma tissue slide and (B) normal image of tumor tissue.

tissue or living animal is necessary for understanding movement, colonisation and growth of tumor cells after application. Recently in the field of cancer biology a tumor cellular marker – the gene encoding GFP is being used. This gene is cloned from *Aequorea victoria* and it is suitable because of high expression, resolution and strong signal (Fig. 1).

We tested the ability of BP6 cells transfected with GFP to induce tumor process in Wistar rats (58 total, 25 male/33 female). Overlay of cells indicated the expression and presence of gene for GFP in almost 100 % tumor cells. The animals were injected by tumor cells at 36–39 days of age by two doses of cells: 0.5 ml and 1.0 ml tumor cells/2 ml nonplasma medium. In view of our planned experiments we performed application of tumor cell into intraperitoneal (IP) and subcutaneous (Sc) area. So each sex were divided into four groups – IP 1.0; IP 0.5; Sc 1.0 and Sc 0.5. Successfulness of intraperitoneal tumor growth in male of group IP 1.0 was 14.3 % (1/7) and in IP 0.5 group 28.6 % (2/7). However, the presence of subcutaneous tumors in male we detected in 75 % for Sc 1.0 (3/4) and none for Sc 0.5 (0/7). In female the intraperitoneal application was prosperous in 28.6 % (2/7) for IP 1.0 group and 11 % (1/9) for IP 0.5 group. Similarly,

in the subcutaneous group of female rats, higher occurrence of subcutaneous tumor – in group Sc 1.0 was 71.4 % (5/7) and 40 % in Sc 0.5 group was seen (Fig. 2).

Acquired data proved that BP6 cells incorporated GFP gene have smaller ability to induce both experimental intraperitoneal and subcutaneous tumor process in host organism. Higher incidence of experimental tumorigenesis, reflected by higher dose of cells indicates that these cells are possibly less resistant to immune response of organism. However we also supposed that incorporation of GFP gene might impair physiological properties of cytoskeleton and worsen adhesive characteristics of tumor cells. In consequence, tumor cells have a large intraperitoneal area and more limited successful cell division than in subcutaneous area.

New possibilities to use GFP in experimental tumorigenesis and clinical medicine

Even before discovery of GFP, in 1955 Davenport and Nicol published work about capability of photocytes *Aequorea victoria* and other jellyfishes to produce green fluorescence in reaction to lamp with UV light in higher wavelength. Presence of GFP

was later confirmed also in others Coelenterata (14). These authors demonstrated *in vitro* the principle of green emission after disintegration of fluorescence granules in the presence of calcium. Consequential studies concerned mainly about GFP from *Aequorea*. Within the light-emitting cells of the jellyfish *Aequorea*, GFP and aequorin molecules are tightly packed in small membrane-bound photophores. GFP is sensitive to changes in pH, temperature, concentration of proteins, ions, which impair its spectral properties. Polypeptide chain consists of 238 amino acids; it folds into a rigid, 11-stranded β -barrel threaded by an α -helix running up the axis of the cylinder. It is known that fluorescence proves thanks to phenol group in chromophore. Understanding of chemical structure contributed to the study of principles underlying the fluorescence reaction. Surprisingly, the chromophore consists of primary amino acid sequence and during chromophore production no enzymes are needed (6). Translation runs the autocatalytic mechanism giving rise to chromophore from a tripeptide (Ser65-Tyr66-Gly67) and is held essentially in the centre of the can-shaped protein (7).

Isolation of GFP gene opened up entirely new areas of its application. GFP inserts come directly from pelagic jellyfish *Aequorea victoria*, and this gene sequence can be inserted at the beginning, or at the end of gene for target protein. Insertion is realized outside of cell by using a plasmid vector. After transfection of cells with plasmid, it can be monitored e.g. expression, behaviour, activity and localization of target protein. The expression of the GFP gene in transfected cells is also an ideal *in vivo* marker of the cells. GFP integrated into the chromosome and passed on to subsequent generations of cells through cell division. Expression of GFP was favourably implemented in a variety of cells and organisms: bacteria, yeast, eukaryotic cell lines and transgenic mice. In mammalian cells humanized mutant hGFP-S65T is used, that displayed a high expression level (15) and in comparison with wild GFP, it is 35-fold more fluorescent, and much more easily expressed in mammalian cell lines.

Experimental model of induction of tumor process by application of tumor cells enables the possibility to study many mechanisms. This study opens up valuable information in view of complex interactions between host organism and tumor tissue. One of the most important endeavours in tumor biology is to understand how specific types of tumor cells behave in the body. In the study of microscopic cancer, tumour-cell labelling for visualising tumour cells *in vivo* has been developed. For example the *Escherichia coli* β -galactosidase (*lacZ*) gene has been used to detect micrometastases. However, this and other methods for study of cancer dormancy, growth, and colonisation require extensive histological preparation and sacrifice of the tissue or animal. Optical imaging based on fluorescence expression of GFP can resolve problems of methods for real-time imaging and enables study of tumour cells in viable fresh tissue or living animals. Several groups have selected tumour cell lines to stably express GFP at high levels both *in vitro* and *in vivo*. These cells can be transplanted into animals, visualised *in situ* in fresh tissues and also can be detected for possible subsequent colonisation in other organs. A major advantage of GFP-expressing tumor

cells is that imaging requires no preparative procedures, contrast agents, substrates, anaesthesia, or light-tight boxes (16). For study *in vivo* processes in real time is essential to non-invasively visualize tumor cells using intravital microscopy (17), intravital video microscopy (18), whole-body imaging (19) and using viral vectors to label cells *in vivo* (20). Transfection of tumor cells with GFP cDNA produces a heritable, stable cytoplasmic marker that allows cells to be detected for long term observations *in vivo* (21). In the study of the behaviour of tumor cells, GFP expression can be used in many tumor cell lines: for lung cancer (22), prostate cancer (23), melanoma (24), colon cancer (19), pancreatic cancer (25), breast cancer (26), ovarian cancer (27) and brain cancer (28). These studies confirmed that tumour cells transfected with the GFP gene are a powerful tool for *in vivo* visualisation of tumour growth (25), characterization hypoxia in the tumor microenvironment (29), angiogenesis, leukocyte/endothelial interactions, permeability of individual tumor vessels (30), dormancy, dissemination, invasion, and metastasis (31).

Dense interactions between the brain and peripheral tissues create a basis for involvement of the brain in various physiological and pathological processes. The brain receives information from peripheral tissues innervated by sensory nerve endings possessing various receptor types. Recently, a non-traditional sensory systems transmitting information to the brain are proposed. These include for example adrenal medulla cells monitoring metabolic changes in the blood (32) or vagal sensory ending monitoring tissue cytokine levels (33) and plasma catecholamines (34). On the other hand brain might influence activity of almost all cells of an organism, directly or via modification of immune and endocrine system activity. The study of the role of the brain in monitoring and modulation of peripheral tissues and organs functions during physiological and pathophysiological conditions is a part of neurobiological view of diseases (35, 36). Neurobiology of diseases also studies involvement of the brain in monitoring and modulation of tumor growth (37). It is suggested that the brain might influence tumor proliferation by modulation of immune system activity (38).

In our experiments we studied changes in brain areas processing sensory information in tumor bearing rats. Therefore the aim of our pilot studies was to develop appropriate experimental tumor model that will enable us to study interactions between the brain and peripheral tumor. We found that intraperitoneal or subcutaneous application of 0.5×10^6 BP6 fibrosarcoma cells into Wistar rats produces successful induction of solid and relatively easy detectable tumors localized in restricted compartments of an organism. In this animal tumor model changes of immune and endocrine organs and changes in activity of selected brain areas were found (13, 38).

Detection of tumor growth in its early stages is usually difficult. In some cases implantation of BP6 tumor cells did not lead to development of tumors, because of elimination of applied tumor cells by immune system. To determine whether tumor will be induced is usually possible only after longer time period after tumor cells application. Therefore we focused on labelling of BP6 cells by incorporated gene for GFP. GFP gene is integrated

into the chromosome, cell division pass this gene on to subsequent generations of cells. Studies indicate that transduction of GFP does not cause cytopathologic or growth-inhibiting effects in tumor cells (18), gene encoding GFP does not have a negative effect on monitoring of promotor activity (39). This approach is successfully used in many tumor cell lines (22, 23, 24, 26, 27, 31). Our data suggest interference between properties of BP6 cells and GFP. It is looks that BP6 cells with incorporated gene of GFP proliferate in vivo less than BP6 without incorporated gene for GFP. Study comparing the capability of tumor induction in experimental model of colon adenocarcinoma cells transfected with GFP in immunocompetent and T- and B-cell-deficient SCID mice shows that immune reaction was responsible for early elimination of these tumor cells (40). On the immune response of GFP and that GFP acts as antigen indicated also another studies (30, 41, 42). There also exist another opinion that the immune system of experimental animals recognize just virus plasmid as extraneously factor (43). Indeed another question is also properties of individual tumor cell lines. The fact is that in our experiment we used fibrosarcoma tumor cells inducing solid tumors. Studies describing positive experiences with GFP transfected tumor cells are dealing with monitoring of metastatic process (21, 23, 24, 26, 31). Even growth rate of tumor cell lines transfected with GFP and without transfection in tissue culture don't show significant differences (42). But in organism tumors are not simply composed of malignant cells, but are intimately associated with host cells. Tumor cells being contacted by host dendritic cells, macrophages engulfing tumor cells, and lymphocytes attacking the tumor (44). It's evident that induction of tumor growth with GFP cells is result of multilevel interactions within organism.

Conclusion

It is more of suspicion than an argument on the enormous significance of described information and partly of the study observations. Because of great importance of protein molecules we can't repudiate the significance of GFP at the grade of higher regulatory systems than suspension of cell cultures. In view of prospective progress of scientific research we suppose prevalent use of GFP in marking of tumor in organism for host immune system. If GFP would be "a bait and sign" for immune system, then his importance of "imaging of cells" would be changed on complicated marker for other intervention, progenitor cells not excepting.

Shimomura had no idea of the applications of green fluorescent protein for a long time. As he said: "I don't do my research for application or any benefit. I just do my research to understand why jellyfish luminesce, and why that protein fluoresces".

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