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Mini-Review

Safe biotechnology (5). Recommendations for safe work with animal and human cell cultures concerning potential human pathogens

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Abstract. The benefits of using animal or human cell cultures have been clearly demonstrated in diagnostic and therapeutic research and in their application for manufacturing. Cell cultures serve as a tools for the production of vaccines, receptors, enzymes, monoclonal antibodies and recombinant DNA-derived proteins. They represent an integral part of drug development for which corresponding facilities, equipment and manufacturing processes are required. Although the cells themselves offer no particular risk to workers in laboratories and production areas or to the environment, the cell cultures may be contaminated with viruses, mycoplasma, bacteria, yeast and fungi or might contain endogenous viruses. The containment level for animal and human cells is therefore determined by the risk class of these agents. The history of animal and human cell cultures has proved that they can be handled safely. The recommendations in this publication concern the safe handling of cell cultures (tissue explants, primary cell cultures) and permanent cell lines of animal and human origin. A classification system of safety precautions has been elaborated according to the potential for contamination with the pathogenic agents involved.

Introduction

Primary cell cultures have been used for decades in basic research and in the production of vaccines. In recent years permanent cell lines have gained importance for research and for the development and production of active substances for diagnostic and therapeutic use and it has been shown that they can be handled safely. This situation has been recognized, for example, in Japan, where according to Japanese Guidelines (1987) operation of large-scale animal cell culture processes by application of Good Industrial Large-Scale Practice (GILSP) can be approved.

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The terms used for animal and human cell cultures in this publication include:

Type cell culture	Explanation
Animal cell culture	Cultures consisting of animal cells apart from organ cultures
Human cell culture	Cultures consisting of human cells apart from organ culture
Tissue culture	Tissue explant or organ culture
Primary cell culture	Cell cultures grown directly from tissues usually unable to grow beyond a limited number of generations
Permanent cell line	Indefinitely growing (or immortalized) cell cultures obtained by one of the following methods: Specific cell type isolated from cells of tumour origin Mutant derived from primary cells Virus modification of primary cells Recombinant DNA modification of primary cells
Hybridoma cell line	Cell fusions of a primary cell with a permanent cell line

The different steps in establishing and using animal or human cell cultures are illustrated in Fig. 1.

Benefits of using cell culture

Animal cell culture has become one of the most important technologies for research and development in cell biology, virology, immunology as well as in the fields of tumour research, toxicology and pharmacology. The utilization of cell cultures has been essential for the progress that has been achieved in these areas of research. In the last few years there have been new developments in the use of permanent cell cultures for traditional vaccine production. New proteins such as monoclonal antibodies, natural blood proteins and compounds of the hematopoietic, erythropoietic, and immune system have gained therapeutic importance. Many of the novel diag-

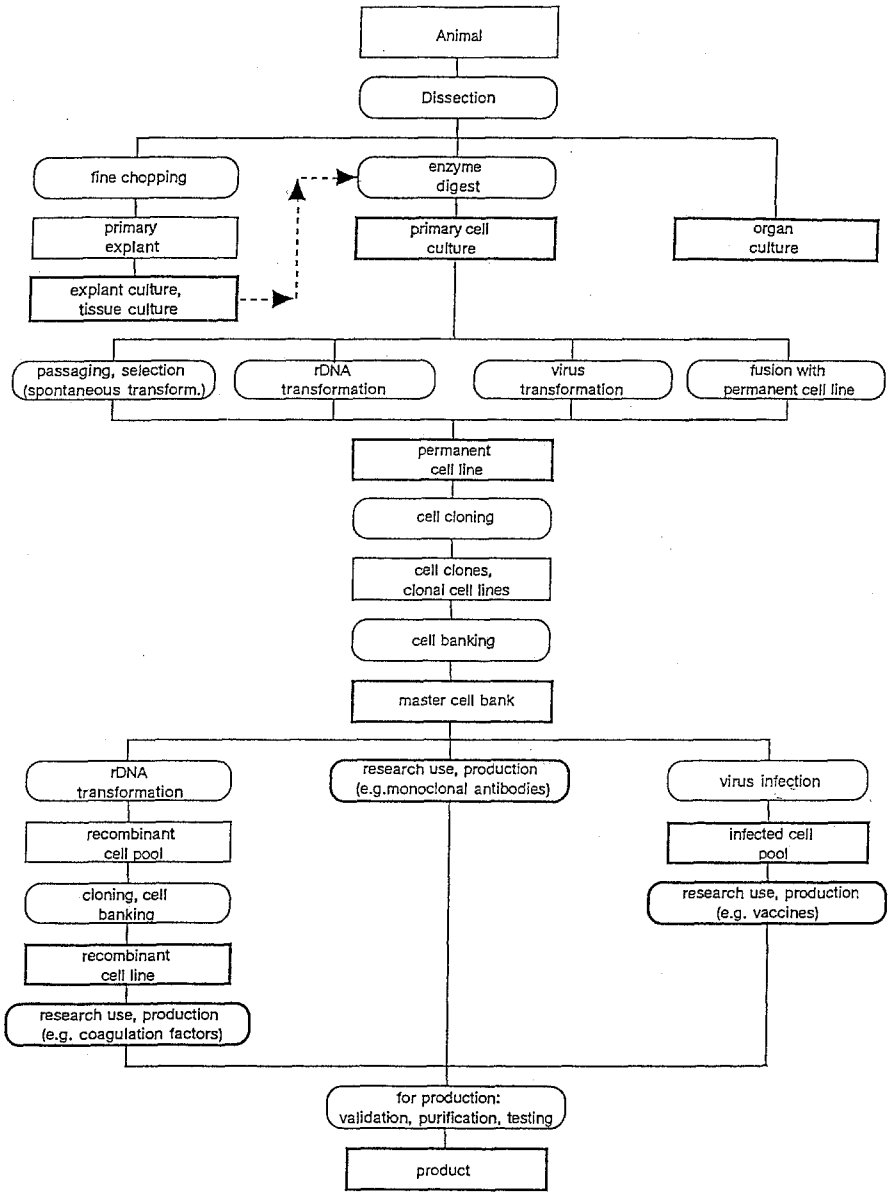

Fig. 1. Establishment and use of animal cell cultures

nostic kits utilize these products, e.g. in pregnancy tests, tumour imaging and for the detection of viral infections.

Although many biologically active proteins are currently produced in microorganisms, a substantial number of the recently discovered and identified products of pharmacological relevance cannot be produced in a physiologically suitable form by bacteria or even by yeasts. The ability of microorganisms to synthesize proteins in their correct secondary, tertiary or quarternary conformation or to glycosylate them in the proper way is limited. In many cases proteins derived from microorganisms have to be chemically refolded and incorrectly folded or processed proteins must be removed. Mammalian cells carry out appropriate post-translational processing during biosynthesis of proteins and peptides. By expressing recombinant DNA in mammalian cell culture, complex and properly glycosylated substances can be produced in their native form and in sufficient quantities.

Potential hazards to humans associated with cell cultures and permanent cell lines

Irrespective of the research opportunities and medical benefits that are obtained by using animal and human cell cultures, there is a need to evaluate potential hazards associated with these cultures. The question of hazards linked to animal and human primary cells and permanent cell lines is raised because cells may harbour endogenous viruses or be contaminated by adventitious agents such as viruses, mycoplasma, bacteria, yeast and fungi.

Hazard assessments are made routinely in laboratories or production facilities. For practical reasons it is desirable to have an evaluation system that is comparable to established approaches for the handling of microorganisms including viruses. The aims of this paper are to harmonize risk assessment and to establish consistent safety measures.

The cells and their DNA

Long-standing experience shows that the cells of cell cultures themselves do not constitute a hazard to man. No cases of tumour development have been reported from accidental lesions during surgical removal of tumours. However, permanent cell lines may cause transplantable tumours in immunosuppressed animals. One case has been reported where a laboratory worker developed a tumour from an incidental needle-stick transmission of a human tumour cell line (Gugel and Sanders 1986). Whether this isolated case has any implication for healthy humans is still doubtful (Hillemann 1979; Petricciani 1987). Apparently, the immune system normally provides a sufficient level of protection whereas humans who are immuno-compromised either by disease or by treatment with immuno-suppressive drugs may be at elevated risk. Experiments with transformed human cells derived from the investigator, such as hybridomas developed from autologous lymphocytes or from lymphocytes genetically modified, also pose a particular hazard, because they essentially circumvent the immune protection system. Such experiments should be strongly discouraged or carried out at a high level of protection for the experimentor concerned.

Tumourigenicity studies seem of no great value in determining the safety of a cell line because most permanent cell lines are tumourigenic in immunocompromised hosts. As pointed out above, recent evidence supports the notion that even tumourigenic cells do not constitute a hazard to healthy humans. Similar considerations are valid for oncogene expression (WHO 1987).

The main hazard inherently associated with cell cultures is related to the expression of latent viruses. Endogenous sequences similar to retroviruses can be found in a number of mammalian species, including humans. Only in special inbred mouse strains that have been bred over decades to create a spontaneously high rate of leukemia (AKR, C57BL-mouse strains) was a pathogenic potential of such sequences reported (Johannsen et al. 1988). Until now it has been possible to activate amphotropic retroviruses only by a chemical treatment of mouse cells. Retroviruses can be detected with the help of new methods such as the polymerase chain reaction (Johannsen et al. 1988). The results may also be used for classification.

Heterogenous DNA of cellular origin present in cell culture fluid might be envisaged to pose a hazard. This subject is reviewed in Annex I of the WHO Report (1987). Available evidence appears to indicate that chromosomal DNA from tumour cells and even cloned activated *ras* oncogenes do not lead to tumour formation when injected subcutaneously. However, DNA from oncogenic viruses (Polyoma, SV40, Rous Sarcoma Virus), if present, increase the hazard of cell-derived DNA.

Contamination by adventitious agents

Adventitious agents are defined as contaminants of animal or human cell cultures such as bacteria, fungi, mycoplasma and viruses. Bacteria and fungi are immediately apparent because they overgrow the cells. Yet mycoplasma and viruses do not necessarily change the appearance of a culture dramatically or lead to cell death. A few examples are given to alert users to the most common contaminations.

Viruses. Human lymphocytes and macrophages may contain agents such as that of hepatitis B virus, human retroviruses, for example human oncogenic or immunodeficiency virus; haematogenous cells from non-human primates may contain simian retroviruses pathogenic for humans. Other simian viruses associated with severe human disease are *Herpesvirus simiae* (Monkey B virus) and Marburg virus. Rodents carrying lymphocytic choriomeningitis virus, Sendai virus, Reo-3 virus and Hantaan virus have led to human disease by direct infection. Caprine and ovine cells can be contaminated with lentiviruses (WHO 1987). There is also evidence that particular treatments (e.g. co-cultivation or exposure of cell cultures to UV light might lead to induction of latent (endogenous or adventitious) viruses.

Mycoplasma. Mycoplasma pneumoniae is known to be pathogenic to humans.

Potential sources of contamination

Three potential sources of contamination can be distinguished: first, the organism from which the cell culture was established and thereby the cell culture itself; second, a later infection introduced during handling; third, the component of animal origin in the cell culture medium, especially serum. Primary cell cultures have a limited lifespan, typically of about 20 passages. The risk assessment has to focus on the pathogen-carrying status of the animal source.

Animal cells may be divided into different classes with respect to their potential for carrying viral agents pathogenic for human beings. Examples can be found in national guidelines (e.g. Medical Research Council of Canada 1980) or recommendations (WHO 1987). The following classification groups cells according to the likelihood of carrying adventitious agents pathogenic to humans. The three classes proposed by WHO (1987) are illustrated by suitable examples, but this does not constitute an exhaustive list.

1. Low likelihood: cells derived from avian and invertebrate tissues
2. Medium likelihood: mammalian non-haematogenous cells such as fibroblasts and epithelial cells
3. High likelihood: blood and bone marrow cells derived from human or non-human primates; human pituitary tissue cells possibly contaminated by the Creutzfeld-Jacob disease agent; caprine and ovine cells especially of neural origin; hybridomas when at least one fusion partner is of human or non-human primate origin. The appearance of bovine spongiform encephalopathy (BSE) suggests that bovine cells of neural origin should be included in this class, although no case has been observed in man (CEC 1991).

Most frequently, human blood cells are used which are derived from registered blood donors and conform to blood bank standards, i.e. the donors must be clinically healthy and must not show antibodies to HBV, HCV or HIV. In the use of such cells, GOSH / GILSP will be sufficient.

The introduction of adventitious agents during handling has to be taken into account and be prevented by appropriate safety measures (Hay 1991). In addition, the only "contact persons" of a cell line are usually well-trained and healthy laboratory workers. Therefore, the risk of contamination with a pathogenic virus or mycoplasma during laboratory work is very low.

Acquired properties of recombinant cell lines

Special consideration should be given to the properties of the vector used for cell transformation (Directives 90/219/EEC and 90/679/EEC). If it is a defective virus, the considerations on the presence of helper viruses detailed below should be taken into account.

Special care should be taken in the evaluation of containment levels for experiments that may either enhance pathogenicity by insertion of a host oncogene or extend the host range of viral vectors by introduction of novel control elements under conditions that permit a productive infection. In such cases serious consideration should be given to raising the physical containment by at least one level.

Products derived from animal or human cell culture

Although not an issue particular to risk assessment of cell cultures, by analogy with chemical and conventional biotechnological production, the product expressed in cell lines must also be taken into consideration in assessing the hazards of a cell culture process. For the assurance of product quality and safety the corresponding regulatory guidelines have to be followed (e.g. FDA Guidelines, 1987b and CEC Guidelines 1987, 1988).

Safety precautions

General precautions for humans

The potential hazards to humans associated with animal or human cell cultures can be assessed and appropriate safety precautions can be established for both laboratory work and large-scale operation. Because the potential hazard of cell cultures is associated with endogenous or adventitious agents such as viruses, mycoplasma, bacteria, yeast and fungi, risk assessment is based on the probability of the presence of such agents. This particular probability can be reduced either by the utilization of well-characterized sources of animal or human cell cultures and/or by an appropriate set of analytical techniques for characterization of the cell cultures and the products derived thereof. Well-characterized sources of animal cells are gnotobiotic animals, specified-pathogen-free (spf) donors or animals which have passed regulatory quarantine cycles.

Media components are autoclaved as far as possible or sterilized by filtration and, especially in production, biological components are screened for adventitious agents by the manufacturer or by the customer. Products of bovine origine should be supplied from countries where no case of BSE has been reported. The customer should audit the supplier at a regular and well-defined schedule to ensure compliance with relevant law and industrial standards.

Table 1. Safety measures concerning potential human pathogens for the handling of animal cell cultures and bulk product solutions; additional safety measures may be necessary for animal pathogens

Level of characterization	Safety measures		
	Cell culture		Downstream processing
	Likelihood of contamination with human pathogens		Supernatant, process fluids and culture product
	High	Medium or low	
Uncharacterized	CC2/CC3[b]	CC1	Same as for cell culture
No indication of presence of human pathogens[a]	CC1	GOSH/GILSP	Same as for cell culture
Absence of human pathogens verified by testing	GOSH/GILSP		Same as for cell culture
Known human pathogen present	According to risk class of human pathogen		Same as for cell culture
Treated by validated means in order to eliminate or inactivate pathogens	Not applicable		GOSH/GILSP

CC1, CC2, CC3, Containment Category 1, 2, 3; GOSH, Good Occupational Safety and Hygiene; GILSP, Good Industrial Large-Scale Practice

[a] E.g., specified-pathogen-free (spf) origin; extended history of safe use

[b] according to the expected contaminant

Table 2. Relationship between safety precautions and risk classes of microorganisms

OECD	EFB
GILSP	Harmless Microorganisms (EFB Class 1)
Objective: to safeguard hygiene for work with harmless microorganisms that do not require containment. Hygienic process and equipment are used to prevent the contamination of culture or product. GOSH principles are applied throughout, by: 1. Formulating and implementing local codes of practice for safety of personnel, and adequate control of hygiene and safety measures. 2. Adequate written instructions and training of personnel to keep the work place clean and tidy, and to keep exposure to any biological, chemical or physical agent to the lowest level that is reasonably practicable. 3. Providing changing and hand washing facilities as well as protective clothing to be worn during work. 4. Not allowing eating, drinking and smoking at the work place.	This class contains microorganisms that have never been identified as causative agents of disease in man and that offer no threat to the environment. The overwhelming majority of microorganisms used in process biotechnology fall into this class.
Containment Category 1 (CC1)	Low Risk Microorganisms (EFB Class 2)
Objective: to minimize the release of low risk microorganisms. Measures in addition to GOSH: 1. Good Microbiological Techniques (GMT, see Table 3) are to be followed. 2. Floors, ceilings and doors of facilities should be easily cleaned and disinfected. 3. Contaminated by-products and utensils are to be autoclaved or disinfected before cleaning. 4. Aerosol creating procedures must be controlled and contained. 5. Restricted access to the facility.	This class contains microorganisms that may cause disease in man and might therefore offer a hazard to laboratory workers. They are unlikely to spread in the environment. Prophylactics are available and treatment is effective.
Containment Category 2 (CC2)	Medium Risk Microorganisms (EFB Class 3)
Objective: to prevent the release of medium risk microorganisms from primary containment during regular operation. If an accident occurs, this can effectively be dealt with. In addition, effective medical treatment is available if needed. Measures in addition to Category 1 conditions: 1. Only authorized personnel is admitted to the facility. If vaccines are available, personnel is vaccinated. 2. If air is extracted from the facility, HEPA filters are used. 3. Effluents from the facility must be decontaminated or sterilized. 4. An autoclave should be within the facility. 5. All processes involving medium-risk microorganisms must be carried out in hermetically sealed equipment or biosafety cabinets. 6. Protective suits, closing at the back, have to be worn by personnel. 7. Hands and forearms should be washed and disinfected at regular intervals. No jewellery must be worn.	This class contains microorganisms that offer a severe threat to the health of laboratory workers but a comparatively small risk to the population at large. Prophylactics are available and treatment is effective.
Containment Category 3 (CC3)	High Risk Microorganisms (EFB Class 4)
Objective: to prevent release of high risk microorganisms even in the case of escape from the primary containment. This includes full secondary containment which prevents the escape of microorganisms in case there is a breach of primary containment. Measures in addition to Category 2 conditions: 1. Visitors should not be admitted. 2. Facility must be completely isolated. 3. The rooms for complete change of clothes must include an air lock facility with compulsory shower. 4. Negative pressure must be maintained in the facility and the air ducts be protected by double HEPA filters. 5. All effluents must be sterilized. 6. An autoclave must be within the facility. 7. Materials containing high risk microorganisms must be absolutely separated from workers. 8. Protective suits for single use must be worn.	This class contains microorganisms that cause severe illness in man and offer a serious hazard to laboratory workers and people at large. In general, effective prophylactics are not available and no effective treatment is known. It is very unlikely that microorganisms from this class will ever be used on a large scale.

OECD, Organisation for Economic Co-operation and Development; EFB, European Federation of Biotechnology; HEPA, High Efficiency Particulate Air

Table 3. Good Occupational Safety and Hygiene (GOSH); Good Microbiological Techniques (GMT)

Principles of GOSH	GMT for the safe handling of microorganisms with risk potential
1. Local codes of practice for safety of personnel should be implemented, including instruction and training of personnel to keep the workplace clean and to keep exposure to biological, chemical and physical agents to the lowest level that is reasonably practicable. 2. Working clothes are recommended in order to avoid contamination or pollution of personal clothes. 3. There should be no contact between materials or tools in the working place and the mouth of the operator. Eating, drinking and mouth pipetting are not allowed. 4. Staff wash hands after handling living organisms and prior to leaving the laboratory. 5. Working surfaces are disinfected daily as well as after spillage of living organisms. 6. All operations are carried out in such a manner that the creation of aerosols is limited. 7. Solid and liquid waste is disinfected prior to disposal.	1. The operators should have basic knowledge of microbiology. Spreading of pathogens should not occur, e.g. via contaminated surfaces, hands or clothes. All workers should be aware of the risks of cultivated pathogens to people in the vicinity. Entry to the working place should be confined to persons who are aware of these risks. 2. Working clothes have to be worn by personnel to prevent spreading of pathogens. 3. There should be no contact between materials or tools in the working place and the mouth of the operator. Eating, drinking and mouth pipetting are not allowed. 4. Hands should be disinfected after normal work or after spillage of infectious material. 5. Working surfaces and tables should be disinfected after normal work as well as after spillage of infectious material. In the case of spillage of infectious material floors have also to be disinfected. 6. No activities that may produce aerosols are permitted at the working place: blending, filling of bottles or tubes should be carried out in a biosafety cabinet. 7. Reliable equipment should be used. 8. Infected waste should be placed in sealable containers, the outside of which should be disinfected before transport to the autoclave or incinerator. 9. Heat or chemical sterilization processes should be investigated beforehand to ensure that the required killing rate is obtained. 10. In case of accidents, an emergency scheme with details of first aid, cleaning and disinfections should be available, and staff should be trained accordingly.

Criteria for classification of cell cultures as well as for bulk products derived thereof are given below and summarized in Table 1. These correspond to established systems for safety measures for handling of microorganisms as published by the European Federation of Biotechnology (EFB) (Frommer et al. 1989) and in the recommendations of the OECD (OECD 1986) (Table 2).

Animal or human cells that originate from well-characterized sources or that are identified as being free of adventitious agents and endogenous viruses by an appropriate set of analytical techniques require only basic hygienic measures during handling. The intention of these principles of Good Occupational Safety and Hygiene (GOSH), during work in a laboratory and also as part of GILSP in large-scale operations is to limit discharges of viable organisms from research and production facilities. Good Microbial Techniques (GMT, Frommer et al., 1989) are applicable to microorganisms with risk potential and therefore specify more stringent measures than GOSH. A detailed comparison is given in Table 3.

Operations with uncharacterized animal or human cell culture have to be carried out at least under Containment Category 1 (CC1) conditions, equivalent to those used for low risk organisms. If uncharacterized animal or human cell cultures with a high likelihood for the presence of adventitious agents of higher risk classes are used, higher Containment Categories are recommended. The classification of cell fusion experiments and hybridoma cells should be based on the fusion partners. In the majority of cases mouse spleen cells are fused with murine myeloma cells. To classify this cell culture work as GOSH/GILSP, as is usual in most laboratories, the mouse colonies and myeloma cells should be tested on the absence of specific human pathogens (see above).

It should be noted that a cell line containing a recombinant DNA molecule or RNA molecules derived thereof, which contain less than two-thirds of the genome of any eukaryotic virus, may be considered defective and is exempt from any guidelines (NIH 1986). For experiments to be carried out under GOSH and GILSP, it must be shown that the cells lack the helper viruses for the specific families of defective viruses being used. Otherwise the containment level should correspond to the risk class of the intact infective virus, but at least CC1 has to be followed.

Experiments involving infectious animal viruses or defective animal viruses in the presence of helper viruses have to be performed according to the risk class of the viruses involved, i.e. class 2 viruses under CC1, class 3 viruses under CC2 and class 4 viruses under CC3. This is in accordance with the NIH guidelines (NIH 1986).

Precautions for production

Biotechnical production of diagnostic or therapeutic products starts with the creation of a master cell bank (MCB) and/or a manufacturer's working cell bank (MWCB). These cell banks have to be characterized extensively according to the guidelines (FDA Guidelines, CEC Guidelines 1987, 1988). These characterizations include microbial contamination, virus contamination, identity of the cell line, genetic stability and in case of genetically modified cells verification of the genetic construct of the cell bank. From this information the safety

measures for production can be obtained, as given in Table 1.

Permanent cell lines are preferentially used for the production of pharmaceuticals. Wherever possible, only cell lines that are free of adventitious agents and endogenous viruses should be used for production. Therefore, experiments aiming at production should be carried out only in cell lines characterized in that way. If contamination cannot be avoided, for example because many rodent cells contain retroviruses, the purification process should be designed to inactivate or eliminate viruses and be validated by spiking experiments.

The viral clearance factor obtained from the validated downstream processing procedures gives an additional safety aspect if the culture is accidentally contaminated by viruses from laboratory workers or by media components.

Conclusions

The evaluation and assessment of the risks associated with animal or human cell cultures (primary cell cultures and permanent cell lines) and their products is based on the probability of the presence of adventitious agents and of endogenous viruses. This assessment allows an appropriate containment classification to be recommended (Table 1). The safety precautions already described for work with microorganisms can be equally well applied for work with animal or human cell cultures and suffice for the protection of humans.

Note added in proof. After preparation of this paper, an article came to our attention (Burns P. A., Jack A., Neilson F., Haddow S. and Belmain A. (1991). Transformation of mouse skin endothelial cells by direct application of plasmid DNA encoding the human H-ras oncogene. Oncogene 6:1973–1978) which reports that the application of the human H-ras oncogene to mouse skin cells has led to tumor formation.

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