Immortalisation of Ovarian Granulosa and Theca Cells of the Marmoset Monkey *Callithrix jacchus*

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Summary
In view of the increasing need for laboratory primates in biomedical research it is desirable to develop appropriate primate-specific cell culture models that could prevent or significantly reduce the increasing use of primary cultures and experiments with living animals. Follicular granulosa and theca cells are essential for the control of hormone-dependent processes such as the ovarian cycle and pregnancy, but also for the occurrence of hormone-dependent diseases. For this reason it is of great interest to know more about control mechanisms existing in these follicular cell types and the effect of pharmacological or toxicological agents on them. An immortalisation protocol for the two ovarian cell types of the marmoset monkey (Callithrix jacchus) has been developed. All cell lines established so far were examined with regard to the maintenance of known, tissue-specific features (e.g. hormone responsiveness and enzyme expression). The results obtained indicate that it is worth while cloning and characterising the cell lines in more detail so that they could be used after an adequate validation as a defined test system both for basic research as well as for pharmacological or toxicological screening.

Zusammenfassung: Immortalisierung von ovariellen Granulosa- und Thekazellen des Weißbüschelaffen
Um einem steigenden Bedarf an Primaten als Versuchstiere entgegenzuwirken, ist es wünschenswert, geeignete primatenspezifische Zellkultursysteme zu entwickeln, die die zunehmend zu erwartenden Primärkultursysteme und Experimente am lebenden Tier verhindern oder erheblich reduzieren können. Granulosa- und Thekazellen sind essentiell für die Steuerung hormonabhängiger Prozesse wie z. B. Menstruationszyklus und Schwangerschaft, aber auch für die Entstehung hormonabhängiger Erkrankungen. Darum besteht ein großes Interesse daran, mehr über Kontrollmechanismen in diesen Zellen und über pharmakologische und toxikologische Effekte auf diese Zellen zu wissen. Es wurde ein Protokoll für die Immortalisierung der beiden Zelltypen aus den Eierstöcken von Weißbüschelaffen (Callithrix jacchus) entwickelt. Alle bisher entstandenen Zelllinien wurden auf Erhaltung bekannter gewebstypischer Eigenschaften (z.B. Hormonempfindlichkeit und Enzymausstattung) untersucht. Die bisher gewonnenen Ergebnisse deuten darauf hin, daß es sehr lohnenswert ist, einige der Zelllinien zu klonieren und detaillierter zu charakterisieren, so dass sie nach entsprechender Qualitätskontrolle sowohl in der Grundlagenforschung als auch für das pharmakologische und toxikologische Wirkstoffscreening als definiertes Testsystem eingesetzt werden können.

Keywords: 3R, New World Monkey, ovary, immortalisation, in vitro system

1 Introduction

During the last decade there has been a continuous increase in the total number of primates used as laboratory animals. This is stated for example by the Annual report on the use of laboratory animals, edited by the German government (Tierschutzbericht 2001 des deutschen Verbraucherministeriums, http://www.verbraucherministerium.de/tierschutz). Data about primates were selected from this report and compiled in Figure 1 which indicates that the increase is caused mainly by rising numbers of primates needed for drug testing.

A further increase might also be expected with continuous advances in new fields such as functional genomics. Drug screening is a field where substantial progress has been made in the development of suitable cell culture alternatives over the past years (Marx, 1996; Spielmann, 1996). Unfortunately this statement does not pertain to systems using cells from nonhuman primates. From these animals only primary cultures are in use which provide limited cell numbers and the standardisation of which is hard to achieve. It is now time to prevent further experiments with primary cultures by the development of defined, differentiated permanent cell lines.

For biomedical studies in the field of reproductive biology the use of primate models is indispensable, because in this respect many physiological differences are evident in primates compared to other mammalian species. Moreover, human tissue from healthy, pharmacologically unimpaired ovaries is not readily available in most cases. Animal models like the marmoset monkey (Fig. 2) can provide tissue of defined physiological origin.

This small New World monkey is a widely used model species especially in

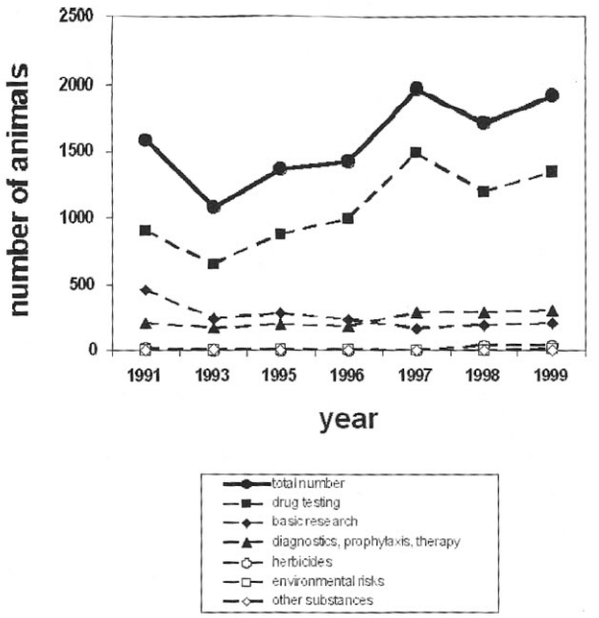

Fig. 1: Number of primates used for animal experiments in Germany since 1991. Data are derived from the Annual report on the use of laboratory animals 2001 (Tierschutzbericht 2001 des deutschen Verbraucherministeriums).

Fig. 2: The marmoset monkey *(Callithrix jacchus)* is a popular primate model in reproductive biology research (Photograph: Courtesy of Alexander Schneiders).

reproductive biology research (Fraser and Lunn, 1999). It is characterised by a high fecundity and a short generation interval. Ovarian anatomy and physiology, including hormonal profiles during the ovarian cycle show a strong similarity to the human (Hearn, 1983; Hillier et al., 1988). Furthermore, the marmoset monkey is the primate model of choice in many toxicological studies (Smith et al., 2001).

The development of permanent cell lines as a defined primate-specific *in vitro* system for research on reproductive biology could serve several purposes. In the first place, it would facilitate studies on primate (including human) ovarian function, such as hormone synthesis or oocyte maturation, as well as studies on hormone dependent diseases of the primate ovary. In the fields of pharmacology or reproductive toxicology it would be a more predictable test system than others using rodent tissues, either *in vivo* or *in vitro*. Furthermore, the evaluation of different immortalisation protocols would help to find general strategies for the immortalisation of cells derived from New World Monkeys, which has not been tried before and might be of interest for the establishment of cell lines from other tissues.

Granulosa cells (GC) and theca cells (TC) form the inner and outer layer of the ovarian follicle wall (Fig. 3). In the marmoset monkey during the course of every ovarian cycle up to three follicles are selected to reach the mature stage of a preovulatory follicle. Only these preovulatory follicles are capable of hormone synthesis in response to a gonadotrophic hormone stimulus from the pituitary. After the hormonal induction of ovulation GC and TC of the preovulatory follicles undergo a terminal differentiation to form the *corpus luteum*. This process is accompanied by endocrine and morphological changes and is called luteinisation. It is a prerequisite for the implantation of the conceptus and maintenance of early pregnancy. GC and TC from preovulatory follicles have to be used to study the changes accompanying luteinisation.

In order to establish a permanent cell line, primary cells have to be immortalised, i.e. their limited life span as regular somatic cells has to be extended (reviewed by Hopfer et al., 1996). Immortalisation can take place spontaneously, by certain mutations which occur often in rodent cells, but only rarely in human and presumably only rarely in other primate cells. Immortalisation can also be achieved experimentally by transfection (transfer of a foreign DNA) or by viral infection with certain oncogenes. Oncogenes code for proteins that deregulate the control of cell proliferation. Some authors reported recently on successful immortalisation by transfection with the catalytic subunit of the enzyme telomerase as an additional method (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Morales et al., 1999). In the project presented here the classical oncogene approach was chosen, i.e. immortalisation by transfection of the well-characterised large T antigen DNA of simian virus 40 (SV40LT). The corresponding protein is known to be targeted to the cell nucleus. There it is thought to bind and inactivate certain antioncogenes which code for proteins that ensure a correct cell cycle control and proliferation (Fig. 4).

After the selection of an appropriate immortalising gene, a suitable technique has to be found to transfer this DNA into the cells. Classical methods utilising agents such as calcium phosphate or the polycation DEAE suffer from the disadvantage of being rather cytotoxic. Meanwhile several reagents are commercially available that work more gently and allow for transfection even in the presence of serum in the cell culture medium.

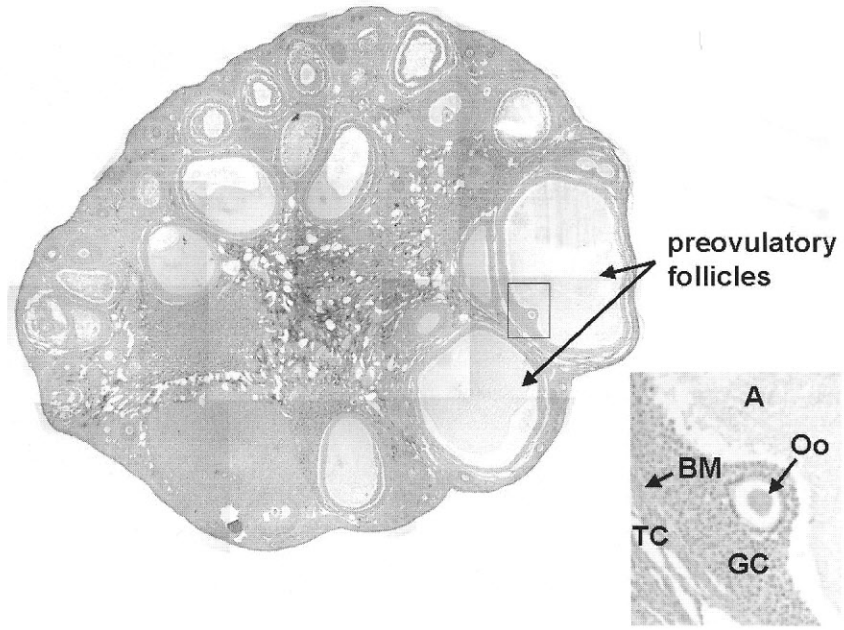

Fig. 3: Microscopic anatomy of a marmoset monkey ovary shortly before ovulation (day 7 of the follicular phase of the ovarian cycle). Two antral, preovulatory follicles are visible. Inset: higher magnification view of the frame marked on one of the preovulatory follicles. A = follicular antrum, GC = granulosa cell layer, TC = theca cell layer, Oo = Oocyte, BM = basement membrane. Hematoxylin-eosin staining, 20x magnification.

A common consequence of the immortalisation of primary cells is that they dedifferentiate and undergo oncogenic transformation, i.e. they lose their former cell type-specific features. For this reason immortalised cells have to be characterised intensively to reveal conformity or emerging differences compared to the corresponding primary cells. Characterisation studies in the present report have focussed on cell-type specific steroid hormone receptor expression and the *in vitro* induction of enhanced progesterone secretion, a characteristic endocrine consequence of luteinisation *in vivo*.

2 Animals, materials and methods

2.1 Animals

Adult, 3-6 year old marmoset monkeys were maintained in family groups at the German Primate Centre, Göttingen, under standardised conditions (Einspanier et al., 1994). All animals had been trained to be handled for routine blood collection. Blood samples were retrieved twice a week over a period of three months for assessment of serum progesterone levels by a specific enzyme immunoassay (Heistermann et al., 1993). This was done to check for regular ovarian cycles and to determine the expected time of ovulation. Three animals were ovariectomised and provided large antral, preovulatory follicles (> 2mm in diameter) for the preparation of primary cultures. Ovariectomy has to be performed regularly for reasons of colony management and was not carried out specifically for the retrieval of GC and TC.

2.2 Vector constructs

Three different plasmid expression vector constructs were used for transfection. Their compositions are schematically depicted in Figure 5.

The vector pEGFP-C1 (Clontech, Heidelberg, Germany) was used for the determination of transfection efficiency. pBluescript-SV40 (Courtesy of Dr. Lisa Wiesmüller, Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Hamburg, Germany, Wiesmüller et al., 1996) and pMSSVLT (Schuermann et

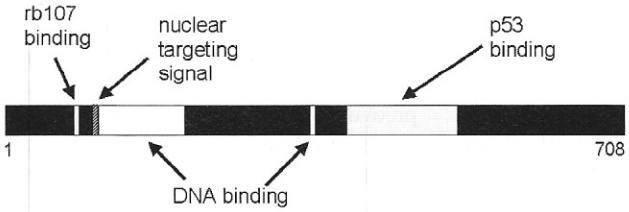

Fig. 4: Primary protein structure of large T antigen of SV40LT and parts of the protein which are thought to be involved in the immortalization process (modified from Modrow and Falke 1998). Rb107 and p53 are cellular antioncogenes and their proper function is disturbed by interaction with SV40LT.

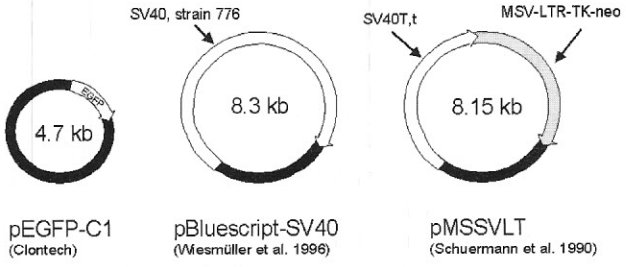

Fig. 5: Vectors used for transfection. pEGFP-C1 codes for enhanced green fluorescent protein. pBluescript-SV40 contains the complete genome of SV40-strain number 776 including the large T region, while pMSSVLT contains only the T-region of the SV40 genome.

al., 1990) include either the complete genome of SV40 or only its large T region, respectively, and were used for immortalisation.

2.3 Cell culture

Primary cultures were prepared according to an established protocol (Einspanier et al., 1995, 1997). Briefly, follicles were punctured and GC are aspirated from the antrum. TC were recovered from the remaining part of the follicle wall and were dissociated mechanically and enzymatically. The cells were washed in culture medium (M199, Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 100 IU/ml Penicillin, 100 pg/ml Streptomycin (Invitrogen Life Technologies). 12-well-culture dishes (Costar, Corning, USA) were precoated with fetal calf serum (FCS). For this purpose, 1ml FCS was applied per well, incubated overnight at 37°C, then aspirated and washed with phosphate-buffered saline (PBS). Primary GC and TC were plated separately on these dishes at a density of 100,000 cells per well. After 48h, the different immortalisation protocols were applied.

Electroporation was performed with Gene Pulser II (BIORAD, Germany). DMEM serumfree, 4mm cuvette 500, 000 cell/ml was tested with vector DNA-concentrations ranging between 1.0 - 5.0 µg.

The protocol for Effectene™ Transfection Reagent (QIAGEN, Hilden, Germany) was optimised according to the manufacturers recommendation.

After transfection the cells were subcultured in untreated dishes, in DMEM (Invitrogen Life Technologies), supplemented with 10% FCS (PAA Laboratories, Linz, Austria). A volume of 2/3 of the culture supernatant was aspirated and replaced by fresh medium three times a week. When confluent, the cells were passaged with 0.025% trysin/0.01% EDTA (PAA Laboratories). Stock cultures were grown in T75CN tissue culture flasks (Sarstedt, Nümbrecht, Germany).

The developing cell lines were named according to the cell type (G = granulosa, T = theca), animal number, immortalising agent (SV = complete genome of SV40 or LT = only large T-antigen from SV40) and line number. For example, line G1SV1 originates from granulosa cells of animal 1, transfected with complete genome of SV40.

For the preparation of genomic DNA or RNA cells were trypsinised and centrifuged according to standard protocols (Freshney, 1994). They were washed with PBS and the final pellet was stored frozen.

2.4 Nested PCR

Genomic DNA was prepared with the DNeasy Mini Kit (QIAGEN). Oligonucleotide primers were designed to amplify a 699 bp fragment of the large T-DNA-sequence in the first round and an internal 400 bp fragment in the second round (see scheme in Fig. 8 and Weber et al., 1994). In each round, 25 PCR cycles were performed with Taq DNA-polymerase using an annealing temperature of 60°C. For amplification with the second primer pair the PCR-product of the first round was diluted 1:100.

Genomic DNA from SVGp12 cells (ATCC-Nr. CRL-8621) which are known to contain SV40LT served as positive control. Non-transfected cells served as a negative control.

2.5 Assessment of progesterone secretion

For stimulation experiments cells were seeded at a density of 100,000 cell/ml/well in DMEM/1% FCS. They were allowed to adhere overnight. Then, 8-bromo-adenosine 3´-5´-cyclic monophosphate (8-Br-cAMP, SIGMA-Aldrich, Munich, Germany) was added at a final concentration of 1 mM according to experimental requirements. At different points in time culture supernatants were collected for progesterone measurement by enzyme immunoassay (Heistermann et al., 1993). The cells were lysed with 100 µl 0.3N NaOH/1% SDS and used for protein determination (Markwell et al., 1981). Treatments were always performed in triplicates. The experiments were repeated at least twice.

2.6 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared with the RNeasy Midi Kit (QIAGEN) and was digested with DNase (PROMEGA, Mannheim, Germany) for 30 min at 37°C

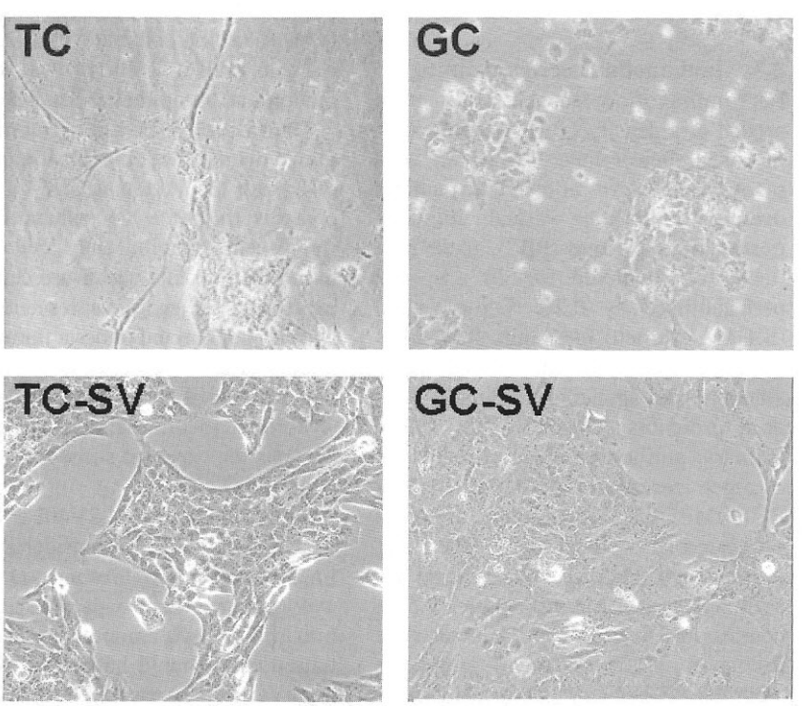

Fig. 6: Primary cultures before and 48h after transfection of pBluescript-SV40 using Effectene™ transfection reagent. GC, TC = untransfected granulosa and theca cells, GC-SV, TC-SV: the same cells after transfection. The transfection protocol itself has no impact on cell morphology.

to remove residual genomic DNA. The synthesis of cDNA was performed using 2 µg of purified RNA with Oligo-dT-Primers and SuperScriptII-Reverse Transcriptase (Invitrogen Life Technologies) according to manufacturers' instructions. From this reaction 1 µl was subjected to PCR analysis.

Oligonucleotide primers for PCR were designed according to the published sequences of the corresponding human cDNAs (estrogen receptor α: Green et al., 1986, forward primer: GTG TAC CTG GAC AGC AGC AA, reverse primer: TCC AGG TAG TAG GGC ACC TG, fragment size: 275 bp; progesterone receptor: Misrahi et al., 1987, forward primer: GGT CTA CCC GCC CTA TCT CA, reverse primer: GGC TTG GCT TTC ATT TGG AA, fragment size: 397 bp). The PCR was performed for 35 cycles at an annealing temperatue of 55°C. Amplification of ribosomal 26S protein mRNA was used as an external standard to prove RNA integrity and loading (Krazeisen et al., 1999; Husen et al., 2001).

PCR-products were visualised applying 10 µl-aliquots from each reaction on 2% agarose gels stained with ethidium bromide.

2.7 Immunofluorescence staining

For immunofluorescence cells were seeded at a density of 10,000 cells/well in 8-well-slides (Nunc, Wiesbaden, Germany) and were allowed to adhere overnight. Cells were washed with phosphate-buffered saline (PBS), fixed with 0.4% formaldehyde for 20 min and permeabilised by 0.2% Triton-X 100 (SIGMA-Aldrich) for 3 min. Antibodies were diluted in PBS/3% bovine serum albumine and applied according to standard protocols (Husen et al., 1999). Primary antibodies were directed to estrogen receptor (ER-B10-A, 1:100, EUROMEDEX, Strasbourg, France) progesterone receptor (PR10A9, Immunotech, Marseille, France) and 3β-hydroxysteroid dehydrogenase (1:500, courtesy of Dr. Ian Mason, University of Edinburgh, U.K.). Secondary antibodies were ALEXA Fluor® 546 rabbit anti-mouse IgG (1:500, Molecular Probes/MoBiTec, Göttingen, Germany). In negative controls the primary antibody was replaced by non-immune IgG.

Staining was visualised with an Axiophot fluorescence microscope using Openlab 3.0™ digital image analysis (Improvision, Coventry, U.K.)

3 Results

3.1 Immortalisation

For the establishment of permanent granulosa and theca cell lines, preovulatory follicles were obtained from three fertile marmoset monkeys shortly before the expected onset of the gonadotropin surge that is preceding ovulation. Primary cultures of granulosa and theca cells were prepared and subjected to different immortalisation protocols.

For the transfer of different vector constructs into the cells we first tried electroporation, but this technique proved lethal in all attempts. Cells were no longer able to adhere to the culture substrate after this treatment. As a second method of DNA-transfer, Effectene™ transfection reagent was used which consists essentially of non-liposomal lipids. This method was successful. Morphologically no cytotoxic effects were observed (Fig. 6) regardless of the type of vector construct used. Primary cells, as well as cells 48 h after transfection had an epithelial-like phenotype.

Transfection efficiencies were determined by transfection of an aliquot of the cells with the vector pEGFP-C1, which contains the gene for enhanced green fluorescent protein. Only cells which have taken up this vector are capable of expressing the green fluorescent protein and can be detected under a fluorescence microscope. This fraction of the total cell number ranged between 5-10% (Fig. 7). According to the manufacturer this represents the expected success rate in primary cells.

After transfection of SV40LT the cells were subcultured and considered as cell lines according to Freshney (1994). It was assumed that within a period of maximally two months non-immortalised cells should have died. At some stage (after 1-10 passages) most cultures underwent the well-known phenomenon of crisis which is accompanied by a stagnant proliferation. After at least 6 passages, as soon as the cells had grown to sufficient numbers, material for characterisation and cryopreservation of the obtained cell lines was collected. Freezing and thawing did not have an impact on cell viability or growth rate. All cell lines characterised here were past their 17.-35. passage.

3.2 Characterisation

After 8 to 12 months of subculture one GC line and six TC lines yielded enough material for characterisation studies.

The successful internalisation of SV40LT was demonstrated by nested PCR of genomic DNA from the respective cells. Interestingly, in most of the cell lines tested so far, SV40LT is not detectable (Fig. 8). Nevertheless, it has to be assumed that these cells are immortalised, because they clearly have an extended life span (so far, up to one year, up to 40 passages) compared to cells in primary culture (maximally 4 weeks, 3 passages). After nine passages of subculture, one of the theca cell lines with

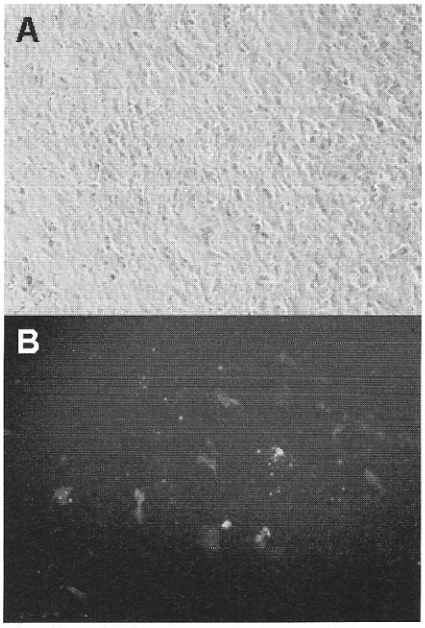

Fig. 7: Determination of transfection efficiency by calculating the fraction of cells that have taken up the vector pEGFP-C1 and correspondingly express green fluorescent protein.
A: Phasecontrast microscopy,
B: Fluorescence micrograph of the same section of the culture dish.

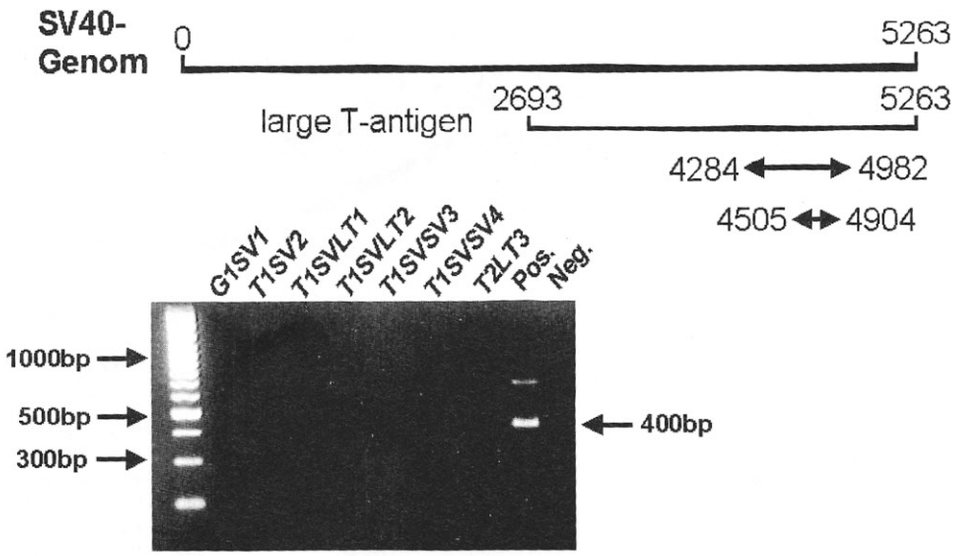

Fig. 8: Demonstration of the uptake of SV40-DNA by nested PCR. Top: Scheme of primer positions and amplified fragments. In this experiment none of the immortalized cell lines tested, but only the positive control cell line SVG contains the DNA-Fragment in question.

no detectable SV40LT (line T1SV2) was subjected to a second transfection, either with the plasmid vector pBluescript-SV40 or with pMSSVLT. In this case no period of crisis was observed with either immortalising vector. Even after this second transfection SV40LT was still not detectable (Fig. 8, lines T1SVLT1, T1SVLT2, T1SVSV3, T1SVSV4).

During luteinisation *in vivo*, granulosa cells are stimulated to produce increasing amounts of progesterone. This process was simulated *in vitro* by incubating the cells with 8-Br-cAMP, a cell membrane permeable analogue of the natural cAMP. This molecule takes part in the physiological signal transduction pathway leading to the stimulation of progesterone secretion after ovulation. Cells cultured without 8-Br-cAMP had a low basal production rate of progesterone. With 8-Br-cAMP progesterone secretion was markedly enhanced within a time period of 48h of culture (Fig. 9).

Before ovulation and luteinisation primate GC express mRNA for estrogen receptor α which mediates cell proliferation in growing follicles via estrogens. Afterwards the expression of this hormone receptor is downregulated (Einspanier et al., 1997, Saunders et al., 2000). Similarly, estrogen receptor expression could be inhibited in immmortalised GC by treatment with 8-Br-cAMP *in vitro* (Fig. 10A). TC have not been tested yet.

Progesterone receptor is expressed *in vivo* only during luteinisation, in GC as well as in TC (Einspanier et al., 1997). In the immortalised cell lines examined a different expression pattern was observed. No progesterone receptor mRNA expression was found in G1SV1 cells with ("luteinised") or without 8-Br-cAMP ("non-luteinised") (Fig. 10A). Instead, progesterone receptor mRNA was detectable in untreated TC where the signal appears to be stronger in those cells which were transfected only once compared to those which were transfected twice (Fig. 10B). Again treatment of TC with 8-Br-cAMP was not yet performed.

Immunofluorescence staining revealed expression of cell type-specific proteins (Fig. 11). The results corresponded closely to the expression of the respective mRNA. Unstimulated G1SV1 cells showed a typical nuclear staining for estrogen receptor α, but not for proges-

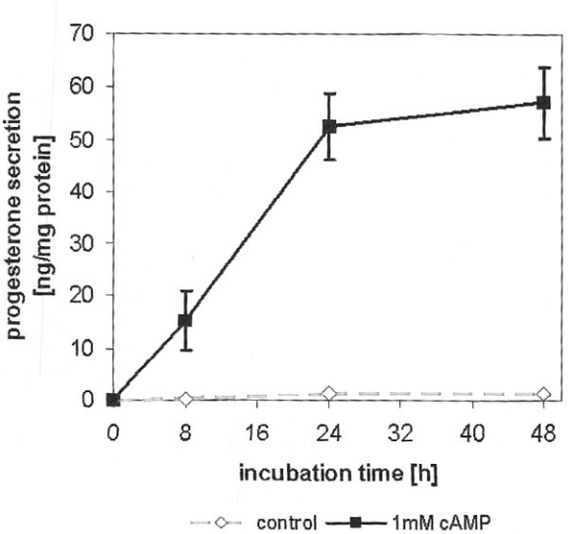

Fig. 9: Stimulation of progesterone secretion in granulosa cell line G1SV1. Cells were incubated with (filled squares) or without (open squares) 8-Br-cAMP for 48h and progesterone content of the culture supernatant was assessed at different points of time.

terone receptor. A moderate immunoreaction in the cytoplasm of the cells was detectable for the progesterone synthesising enzyme 3β-hydroxysteroid dehydrogenase.

4 Discussion

Permanent cell lines from GC and TC of primates would provide an extremely useful experimental test system for biomedical studies. Reproductive physiology of rodents is too different from that of primates including humans. For example, rodents have extremely short ovarian cycles (4-5 days versus 25-40 days in primates, 28 days in humans). Their follicular development is controlled by different regulatory factors. A functional *corpus luteum* is formed only when conception has occurred. Consequently, immortalised rodent cells are not comparable to those of primates. From humans, sufficient amounts of ovarian cells for primary cultures can be obtained only from *in vitro* fertilization (IVF) programs. In this case only luteinised GC are available, because for successful oocyte retrieval the women have to be pretreated with gonadotrophic hormones. Since these luteinised GC behave already like luteal cells, they are not appropriate to study the complex process of luteinisation that is crucial to female fertility. Human TC are not available at all by IVF techniques, although they play an important part in the formation of a functional *corpus luteum* during luteinisation. Only tissue from primates like the marmoset monkey which lives in a controlled environment and in which the ovarian cycle can be monitored exactly meets all requirements of a defined starting material for the preparation of cell cultures.

Many authors have immortalised GC from different species and from different stages of GC development (rat: Amsterdam et al., 1988; Suh and Amsterdam, 1990 and others, pig: Chedrese et al., 1998; Gillio-Meina et al., 2000; human: Rainey et al., 1994; Lie et al., 1996; Hosokawa et al., 1998). The results of the studies in rats and pigs indicate that by immortalisation it is possible to maintain GC at specific differentiation stages cor-

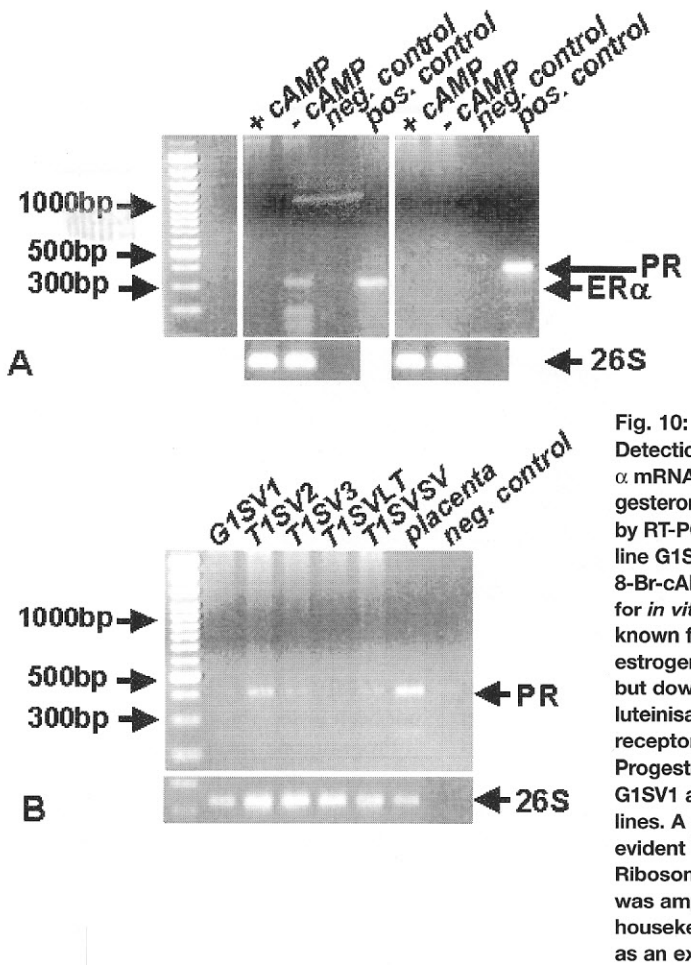

Fig. 10:
Detection of estrogen receptor α mRNA (ERa) and progesterone receptor mRNA (PR) by RT-PCR. A: Granulosa cell line G1SV1 with and without 8-Br-cAMP (cAMP) as a stimulus for *in vitro* luteinisation. As known from *in vivo* experiments estrogen receptor is present, but down-regulated after luteinisation, progesterone receptor is not detectable. B: Progesterone receptor mRNA in G1SV1 and different theca cell lines. A positive signal is evident only in theca cells. Ribosomal 26S protein (26S) was amplified as a housekeeping gene serving as an external standard.

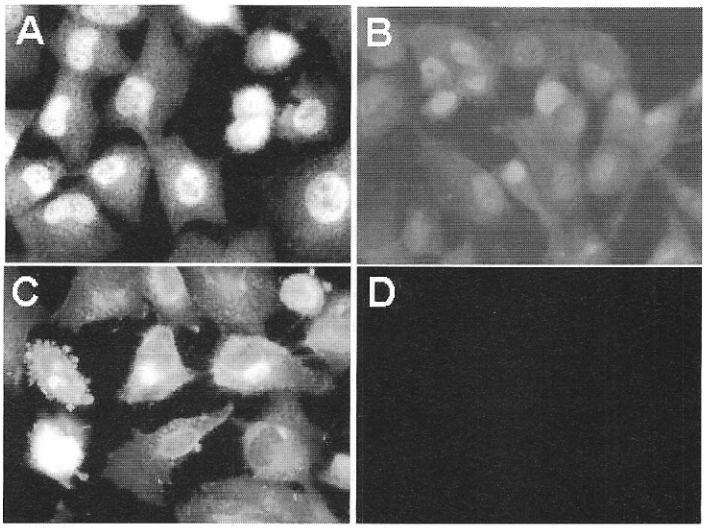

Fig. 11: Immunofluorescence staining of unstimulated G1SV1 cells. As known from the situation *in vivo*, a strong positive immunoreaction is observed for estrogen receptor. (A, nuclear staining) and 3β-hydroxysteroid dehydrogenase (C, cytoplasmic staining), but not for progesterone receptor (B). There is no reaction in the control slide (D, primary antibody replaced by non-immune IgG).

responding to the developmental stage of the follicle they were isolated from. With granulosa cell lines from various stages of follicular development these may be studied and characterised more thoroughly. This is again particularly important for the study of the luteinisation pathway. Similar possibilities should be expexted with theca cell lines. Yet, no one has immortalised TC from preovulatory follicles. None of the ovarian cell lines reported has been validated as a defined *in vitro* test system.

In vivo GC and TC are dependent on each other to fulfil their functions during luteinisation. Their typical enzymes complement each other for proper hormone synthesis. It is important to establish permanent cell lines from both cell types. Only this offers the possibility of exposing one cell type with conditioned medium of the other one and developing cocultures or even organotypic cultures at a later stage of the project.

The results of this study show that it is possible to immortalise primary granulosa and theca cells from New World monkeys by transfection with the classical oncogene SV40LT. Unexpectedly, SV40LT-DNA could not be detected in most of the developing cell lines by nested PCR. In addition, by a quantitative PCR approach with the Lightcycler instrument the expression of the catalytic subunit of telomerase, an enzyme which is known to be expressed in many immortalised cell lines was studied (Harley and Villeponteau, 1995). Again none of the tested cell lines was positive for telomerase (data not shown). The observation of lack of SV40LT contradicts current unterstanding. Thus, the immortalisation mechanism in these cells is still a matter of speculation. It may be suspected that SV40LT protein was expressed from the vectors for some time directly after transfection, so that irreversible interactions with genes controlling cell proliferation were possible.

Some specific features of the newly immortalised cells were assessed. One of the most important functions of GC, the capacity to secrete progesterone could be demonstrated reproducibly. It could be induced by a signalling molecule that is involved in the process of luteinisation *in vivo*. In further experiments other factors involved in the hormonal signalling pathway such as gonadotrophins, steroid hormones or extracellular matrix will be tested.

The mRNA and protein expression of steroid hormone receptors and the progesterone-synthesizing enzyme 3β-hydroxysteroid dehydrogenase were examined as cell-type and differentiation specific markers (Einspanier et al., 1997). This was studied with and without stimulation of progesterone secretion by 8-Br-cAMP. With the exception of progesterone receptor, the expression patterns were exactly as known from *in vivo* experiments (Einspanier et al., 1997; Suzuki et al., 1994). Progesterone receptor expression should have been increased in GC with enhanced progesterone secretion, but this was not the case in cell line G1SV1. The examination of further granulosa cell lines is needed to clarify this issue.

It has to be considered that at the moment the cell lines still represent heterogeneous populations. For the establishment of a defined cell line they have to be cloned and characterised with the same methods again. Generally, it is aimed at developing several cell lines of each cell type, to be able to cover the different phenotypes of these cells that occur *in vivo*. According to the first characterisation studies presented here, the cell lines tested so far, have retained several cell-type specific features. They are a promising basis for the future development of a defined *in vitro* test system.

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Acknowledgements

This work was supported by the foundation SET (Stiftung zur Förderung der Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen). We are grateful to Prof. J. K Hodges for critical reading of the manuscript.

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