

## Integration and expression of the WAP-hPC gene in three generations of transgenic rabbits

### Integrácia a expresia WAP-hPC génu v troch generáciach transgénnych králikov

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**ABSTRACT:** The aim of the present study was to obtain transgenic rabbits expressing human protein C in their mammary gland. A fusion construct which consisted of 4.2 kb long mouse whey acidic protein (WAP) promoter and 9.4 kb genomic human protein C (hPC) was microinjected into rabbit zygotes. Born animals were subjected to PCR amplification to detect the integration of the injected construct into their genome. As examined during three generations, the founders transmitted transgenic allele in a Mendelian fashion. Western blot analysis demonstrated the presence of hPC in the milk of lactating transgenic females. The concentration of the recombinant hPC, as determined by ELISA, ranged between 0.24–0.56 µg/ml. This relatively low expression rate might be caused by the animal species used or might be a subject of position effects of the integration of the heterologous construct.

**Keywords:** WAP-hPC; transgenic rabbits; integration; expression

**ABSTRAKT:** Cieľom práce bolo získať transgénne králiky s expresiou ľudského proteínu C v ich mliečnej žľaze. Génová konštrukcia pozostávajúca z promotora – myšieho srvátkového proteínu (WAP) o veľkosti 4,2 kb a génu ľudského proteínu C o veľkosti 9,4 kb bola mikroinjektovaná do králičích zygot. Integrácia injektovaného konštruktú v génome narodených jedincov bola analyzovaná PCR metódou. Transgénna alela bola v nasledovných troch generáciach prenášaná v rámci Mendelových pravidiel. Western blot analýzy ukázali prítomnosť hPC v mlieku laktujúcich transgénnych králičích samíc. Koncentrácia rekombinantného hPC, na základe ELISA testu, bola zistená v rozpätí 0,24–0,56 µg/ml. Táto relatívne nízka úroveň expresie mohla byť spôsobená živočíšnym druhom alebo mohla byť zapríčinená miestom integrácie heterológneho konštruktú.

**Kľúčová slova:** WAP-hPC; transgénne králiky; integrácia; expresia

Protein C (PC) is a key enzyme in the blood anticoagulation pathway. In humans it is synthesized predominantly in the liver. The hPC consists of a heavy chain (41 kDa) and light chain (21 kDa) linked by a disulfide bond (Kisiel, 1979). In plasma, PC is present at concentrations 10 µg/ml. Deficiency of hPC is inherited as an autosomal dominant disease and it is associated with serious thromboembolic complications. Replacement therapy using a concentrate of hPC was shown to be effective in preventing syndromes related

to this disease (Regnault *et al.*, 1991). However the isolation of hPC from human plasma is very difficult (Velander *et al.*, 1989). Therefore the interest in the development of alternative means for the hPC production, especially in the last decade, has been arising.

Transgenic animals represent an alternative way to produce biologically active proteins playing role in the blood coagulation (Lubon and Paleyanda, 1997). Production of recombinant human protein C (rhPC) was accomplished in the mammary gland of transgenic

pigs (Velander *et al.*, 1992; Lee *et al.*, 1995; Van Cott *et al.*, 1997) and to a lesser extent in the mammary gland of mice (Velander *et al.*, 1991; Drohan *et al.*, 1994). Recently rabbits were shown to be a suitable model for production of the recombinant proteins in their mammary gland (e.g. Strömqvist *et al.*, 1997). Although rabbits are not conventional dairy stock, the short generation cycle and multiple offspring per litter offer advantages for establishment of a herd-producer of important therapeutic recombinant proteins. This species alleviates some disadvantages of large animals, such as pigs and sheep, and small animals, such as mice. Therefore in the present study, we established transgenic rabbits expressing protein C in their mammary gland. Since production of recombinant proteins requires genetic stability of transgene, we investigated hPC expression in three generations of founder's offspring.

## MATERIAL AND METHODS

### Generation of transgenic rabbits

Transgenic rabbits were generated by the microinjection of the WAP-hPC gene constructs which consisted of the 4.2 kb WAP promoter and 9.4 kb hPC gene (Drohan *et al.*, 1994) into the pronuclei of fertilized eggs (Brem *et al.*, 1985) from superovulated New Zealand White female rabbits. Microinjected embryos were transferred to the oviducts through the infundibulum of pseudopregnant recipients.

### Detection of the WAP-hPC gene integration

Integration of the injected construct into the rabbit genome was analyzed by PCR amplification on the DNA isolated either from blood or from ears (Sambrook *et al.*, 1989) or from the embryos (Chrenek *et al.*, 1998). Firstly, for PCR amplification hPC specific primers: 5'-CAG CAC AGC CTC CCC TAC TCA AA-3' and 5'-CTC CGC CCC CTC AAG ACT CAT TC-3' (Chrenek *et al.*, 1997) at the annealing temperature 68°C in 35 cycles were used. Positive samples were re-analyzed using additional hPC specific primers: 5'-CAG TCA CTT GCC TGA CAC CGG TAC-3' and 5'-GCC AGT GTG CAT TTG AGT AGG GGA-3' (Drohan *et al.*, 1994) at the annealing temperature 58°C in 35 cycles. PCR products were visualized using 1.5% agarose gel (Serva, Heidelberg, Germany).

### Milk collection

Milk of the transgenic females or from the control non-transgenic rabbits was collected on the 20th day of lactation. In F<sub>1</sub> generation, milk sample of one transgenic female was collected during the second, third and fourth lactation. In F<sub>2</sub> generation, milk samples of 9 transgenic females were collected and subsequently analyzed. In order to stimulate milk letdown, intramuscular injection of 5 IU of oxytocin (Léciva, Prague, Czech Republic) was applied 15 min before the milk collection. Thereafter, obtained milk was immediately centrifuged at 12 000 g for 10 min and the upper lipid layer was removed. The samples were either subjected to further analyses or stored in liquid nitrogen.

### Western blotting

The defatted milk samples of transgenic and non-transgenic females were separated by SDS-PAGE under non-reduced conditions according to Laemmli (1970). Proteins were then transferred by semi-dry trans-blot (Bio-Rad, Vienna, Austria) onto the ECL Hybond membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The intrinsic peroxidase activity was quenched by the incubation of the membrane in 3% H<sub>2</sub>O<sub>2</sub> for 15 min followed by the incubation in 5% BSA dissolved in TTBS (Tris-buffered PBS – Tween 20, pH 7.5) for 1 h at room temperature. Thereafter, the membrane was treated with sheep anti human protein C primary antibody and subsequently with peroxidase-conjugated rabbit anti sheep IgG secondary antibody (Dako A/S, Glostrup, Denmark). Signals in the membrane were visualized using ECL detecting reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

### ELISA

Detection of the rhPC concentrations in rabbit milk were accomplished by the polyclonal Asserachrom Protein C ELISA kit (Diagnostic Stago, Ansieres, France). Shortly, 200 µl of milk samples were incubated for 2 h into 96-well Immulon plates pre-coated with rabbit anti-human protein C antibody. After a washing step, 200 µl of rabbit anti-human protein C antibody coupled with peroxidase was added and incubated for 2 h at room temperature. All samples were washed again and thereafter incubated with 200 µl OPD/H<sub>2</sub>O<sub>2</sub> substrate for 3 min. The reaction was stopped by addi-

tion of 50  $\mu$ l of 3M H<sub>2</sub>SO<sub>4</sub> and the absorbancies determined at 492 nm were compared with the established standard curve.

### Assay for the biological activity

The biological activity of the rhPC in the milk samples was measured using an activated partial thromboplastin time (APTT) assay (Velandar *et al.*, 1991). The APTT reagent included Protac (Agkistrodon contortrix venom) from Diagnostica Stago, Ansieres, France to specifically activate hPC (or rhPC derived from milk samples added at different dilutions) which inhibits factors Va and VIIIa prior to the addition of CaCl<sub>2</sub> to initiate coagulation. Thus the activation of human protein C results in an increase in the APTT.

## RESULTS

### Generation of transgenic rabbits and detection of the WAP-hPC gene integration

Altogether 140 zygotes obtained from donor rabbits were injected with the WAP-hPC gene construct into the male pronuclei. The microinjected eggs were trans-

ferred into the oviducts of 10 recipients and 30 offspring were born after the transfer.

PCR analysis of the genomic DNA isolated either from blood or from ears (Figure 1) revealed integration of the heterologous gene in one male rabbit born after the transfer (F<sub>0</sub>). This male founder was bred with wild-type (wt) females and transmitted the heterologous WAP-hPC gene (Table 1) into F<sub>1</sub> (4 rabbits – one male and three females of 16 born being transgenic). In F<sub>2</sub> generation, 25 transgenic rabbits of 47 rabbits were born (9 females and 16 males). Several transgenic rabbits were further crossed with non-transgenic counterparts to obtain F<sub>3</sub>. In F<sub>3</sub>, the integration of the heterologous construct was evaluated either by analyzing born rabbits (53 rabbits of 93 rabbits born being transgenic) or by PCR amplification accomplished on the embryos at the blastocyst stage obtained from anesthetized females (46% rabbit embryos being transgenic).

In summary, the transgenic rabbits were apparently normal and crossing of transgenic rabbits with non-transgenic counterparts yielded litters of normal size ( $7 \pm 0.27$ ) with 45% representation of the transgenic allele in 156 analyzed offspring, thus proving its Mendelian genetic distribution. No disturbances in the lactation of transgenic rabbits were observed, however, about 10% of the rabbits in F<sub>1</sub> and F<sub>2</sub> died before reaching their adult age.

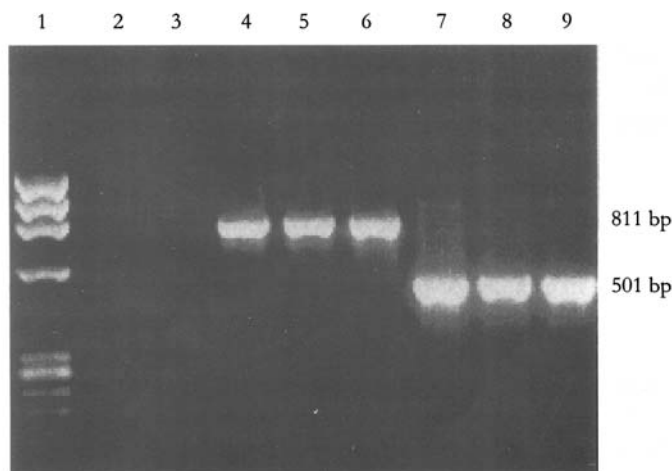


Figure 1. Detection of the hPC gene integration by PCR analyses. The size of the PCR amplified product using primers as described in Material and Methods was 811 bp (lanes 4, 5, 6; lane 2 as a negative control from non-transgenic rabbit) and 501 bp (lanes 7, 8, 9; lane 3 – negative control from non-transgenic rabbit), respectively. Migration of the DNA marker (PhiX 174/ HaeIII) is shown in lane 1

Table 1. Generations of transgenic rabbits

Generation	No. of newborn rabbits	No. of transgenic rabbits	Sex of transgenic rabbits
F <sub>0</sub>	30	1/30 (3%)	1♂
F <sub>1</sub>	16	4/16 (25%)	1♂, 3♀
F <sub>2</sub>	47	25/47 (53%)	16♂, 9♀
F <sub>3</sub>	93	53/93 (57%)	33♂, 20♀

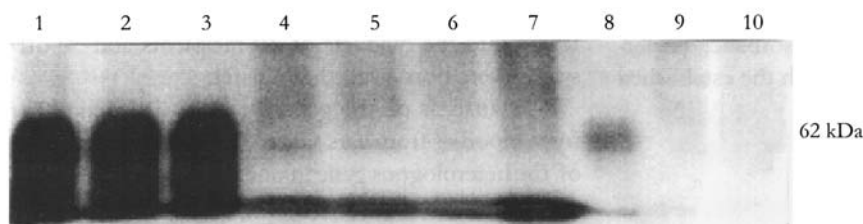


Figure 2. Western blot at non-reduced conditions using sheep anti human protein C antibody and rabbit anti sheep IgG antibody. Detected band of approximate size of 62 kD corresponded to the expected size of the hPC. Lanes 1, 2 and 3 – hPC (2.5 µg, 1.25 µg and 0.62 µg of hPC), lanes 4 – milk from transgenic rabbit of F<sub>1</sub> generation (dilution 1 : 10), lanes 5, 6, 7 – milk from transgenic rabbits of F<sub>2</sub> generation (dilution 1 : 10), lane 8 – human plasma (dilution 1 : 40), lane 9 – milk from non-transgenic rabbit (dilution 1 : 10), lane 10 – milk from transgenic female No. 8 (dilution 1 : 10) incubated with pre-immune serum only

### Detection of the rhPC expression

The presence of rhPC in rabbit milk samples was tested in non-reducing conditions using a sheep anti human protein C antibody followed by incubation with a rabbit anti sheep IgG antibody. In milk samples of transgenic females (Figure 2, lanes 4 to 7) a band of similar size (approximately 62 kDa) as detected in human plasma (Figure 2, lane 8) was observed. The milk sample derived from non-transgenic females (negative control – Figure 2, lane 9) and the milk sample of transgenic female which was incubated with pre-immune serum only (Figure 2, lane 10) showed no specific signal.

The concentrations of rhPC in the milk of lactating transgenic female rabbits determined by ELISA varied between 0.24 and 0.56 µg/ml. In the assay for biological activity the addition of milk from transgenic rabbits resulted in increased APTT by 1.5%.

## DISCUSSION

### Capability of transgene transmission

One of the requirements for transgenic animals used for the production of therapeutic proteins is the stability of transgene transmission being accompanied by a steady secretion of the recombinant protein. In the present study using transgenic rabbits as a model, we demonstrated the transmission of the integrated heterologous construct and the presence of rhPC in the milk of transgenic rabbits during multiple lactation in F<sub>1</sub> generation and in F<sub>2</sub> generations. This is consistent with the report of Van Cott *et al.* (1997), who showed a stable secretion of rhPC secreted in the mammary gland of multiple lines of transgenic pigs over multiple

lactations, being transmitted to offspring in a Mendelian fashion.

### hPC gene expression

In order to establish transgenic rabbits, we used a fusion construct consisting of a 4.2 kb long WAP mouse promoter and a 9.4 kb genomic clone of human protein C gene which was previously used for establishment of transgenic mice and pigs expressing rhPC in their mammary gland. In mice, the use of this construct resulted in the rhPC secretion at values up to 0.7 mg/ml (Drohan *et al.*, 1994) that are much higher than those (0.03 mg/ml) obtained with the WAP-hPC cDNA construct (Velander *et al.*, 1991). In established transgenic pigs this construct provided rhPC expression ranging between 0.1 and 1.8 mg/ml (Van Cott *et al.*, 1997). These expression rates were comparable with those obtained with a 2.6 kb WAP-hPC cDNA construct in transgenic pigs (Van Cott *et al.*, 1997). In our study, the expression rates of rhPC in established transgenic rabbits ranged between 0.24 and 0.56 µg/ml. This relatively low expression rate might be influenced by the used animal species or might be the subject of position effects of the integrated construct.

In established transgenic pigs the anticoagulant activities of rhPC isolated from milk ranged between 70% and 150% compared with the anticoagulant activity of hPC derived from human blood (Velander *et al.*, 1992; Lee *et al.*, 1996), thus indicating that the expressed rhPC is active. The accomplished assay in our study indicates that rhPC expressed in the milk of transgenic rabbits might be active and a relatively slight increase in APTT might be correlated with low rhPC concentration in the milk of transgenic rabbits.

In conclusion, by microinjection of the WAP-hPC gene into the male pronucleus we have produced transgenic rabbits expressing recombinant human protein C in their mammary gland. Our study confirms that rhPC can be steadily secreted over multiple generations and lactations. As the secretion level of the obtained rhPC is very low, expedience of rabbit as a source for the production of human protein C still remains under consideration.

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### REFERENCES

- Brem G., Brenig B., Goodman H.M., Selden R.C., Graf F., Ruff B., Springman K., Hondele J., Meyer J., Winnacker E.L., Krausslich H. (1985): Production of transgenic mice, rabbits and pigs by microinjection. *Zuchthygiene*, *20*, 251–252.
- Chrenek P., Vašíček D., Makarevich A., Bulla J., Oberfranc M. (1997): The frequency of human protein C integration into rabbit genome after microinjection. In: 13th Scientific Meeting of E.E.T.A., Lyon, 1997. (142 abstr.).
- Chrenek P., Makarevich A., Vašíček D., Laurinčík J., B J., Gajarská T., Rafay J. (1998): Effects of superovulation, culture and microinjection on development of rabbit embryos *in vitro*. *Theriogenology*, *50*, 659–666.
- Drohan W.N., Zhang D.-W., Paleyanda R.K., Chang R., Wroble M., Velander W., Lubon H. (1994): Inefficient processing of human protein C in the mouse mammary gland. *Transgenic Res.*, *3*, 355–364.
- Kisiel W. (1979): Human plasma protein C. Isolation, characterization and mechanism of activation by thrombin. *J. Clin. Invest.*, *64*, 761–769.
- Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.
- Lee T.K., Drohan W.N., Lubon H. (1995): Proteolytic processing of human protein C in swine mammary gland. *J. Biochem.*, *118*, 81–87.
- Lee T.K., Bangalore N., Velander W., Drohan W.N., Lubon H. (1996): Activation of recombinant human protein C. *Thrombosis Res.*, *82*, 225–234.
- Lubon H., Paleyanda R.K. (1997): Vitamin K-dependent protein production in transgenic animals. *Thrombosis Haemost.*, *78*, 532–536.
- Regnault V., Rivat C., Pfister M., Stoltz J.-F. (1991): Monoclonal antibodies against human plasma protein C and their uses for immunoaffinity chromatography. *Thrombosis Res.*, *63*, 629–640.
- Sambrook J., Fritsch E.F., Maniatis T. (1989): *Molecular Cloning: A Laboratory Manual*. 2nd ed., New York, Cold Spring Harbor Lab. 1626 pp.
- Strömquist M., Houdebine L.-M., Andersson J.-O., Edlund A., Johansson T., Viglietta C., Puissant C., Hansson L. (1997): Recombinant human extracellular superoxide dismutase produced in milk of transgenic rabbits. *Transgenic Res.*, *6*, 271–278.
- Van Cott K.E., Lubon H., Russell C.H.G., Butler S.P., Gwazdauskas F.C., Knight J., Drohan W.N., Velander W.H. (1997): Phenotypic and genotypic stability of multiple lines of transgenic pigs expressing recombinant human protein C. *Transgenic Res.*, *6*, 203–212.
- Velander W.H., Madurawe R.D., Orthner C.L., Tharakan J.P., Ralston A.H., Strickland D.K., Drohan W.H. (1989): Process implications for metal-dependent immunoaffinity interactions. *Biotechnol. Prog.*, *5*, 119–125.
- Velander W.H., Page R.L., Morcöl T., Russell C.G., Canseco R., Young J.M., Gwazdauskas F.C., Wilkins T.D., Johnson J.L. (1991): Production of biological active human protein C in the milk of transgenic mice. *Ann. N. Y. Acad. Sci.*, *665*, 391–403.
- Velander W.H., Johnson J.L., Page R.L., Russell C.G., Subramanian A., Wilkins T.D., Gwazdauskas F.C., Pittius C., Drohan W. (1992): High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc. Natl. Acad. Sci. USA*, *89*, 12003–12007.

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