



**PRODUCTION AND CHARACTERIZATION OF
TRANSGENIC MICE AND PIGS CARRYING
THE PORCINE GROWTH HORMONE GENE**

by

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SUMMARY.

Studies reported in this thesis examine the possibility of creating transgenic animals with improved growth characteristics by introducing additional copies of the growth hormone gene into their genome.

In the first series of experiments the mouse was used as a model animal to develop the microinjection procedure and to prove the effectiveness of human metallothionein/porcine growth hormone gene construct in stimulating accelerated growth. A number of transgenic mice, produced by pronuclear injection, grew at increased rate and some of them reached almost twice the size of their non-transgenic littermates. These mice were shown to pass the foreign gene and the large phenotype to a portion of their offspring, thus demonstrating stable incorporation of the introduced gene in their genome. In an attempt to obtain better control of the gene expression, transgenic mice carrying modified human metallothionein/porcine growth hormone fusion gene were produced and their growth performance was studied following zinc induction.

The second group of experiments describes production of transgenic pigs with improved growth performance. One of the transgenic pigs created grew at markedly enhanced rate as compared with other transgenic and control animals. Transmission of the introduced gene to second generation of pigs was also achieved. The presented results demonstrate for the first time that production of stable lines of transgenic farm animals with enhanced growth performance is feasible.

The studies aimed at providing the analysis of endocrine and metabolic state of transgenic mice and pigs, their body composition and reproductive capacity are described in the last part of the thesis. Evidence is presented to show that accelerated growth of large transgenic animals is a consequence of the expression of the introduced gene and the production of biologically active porcine growth hormone. The overproduction of porcine growth hormone

results in the increase of plasma concentrations of insulin-like growth factor-1 in both mice and pig, alteration of body composition and changes in metabolic state of mice, and impairment of reproductive capacity of female mice.

DECLARATION.

I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge, no material described herein has been previously published by another person, except where due reference is made in the text.

If accepted for the award of a Ph.D. degree this thesis will be available for loan and photocopying.

Anna E. Michalska

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LIST OF PUBLICATIONS.

Aspects of the work herein have been published as follows:

1. Michalska, A.E., Robins, A.J., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1984) "Microinjection of a chicken histone gene into mouse zygotes". Proc. Aust. Soc. Reprod. Biol. **16**: 108.
2. Michalska, A.E., Robins, A.J., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1985) "Successful gene transfer into mouse embryos by pronuclear injection". Proc. Aust. & N.Z. Soc. Cell Biol. **4**, (Abstr.).
3. Michalska, A.E., Stone B.A., Quinn, P. and Ashman, R.J. (1985) "Effects of pyruvate and lactate on *in vitro* development of one-cell pig embryos". Proc. Aust. Reprod. Biol. **17**: 88.
4. Michalska, A.E., Vize, P., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1986) "Porcine growth hormone gene regulates growth of transgenic mice". Proc. Aust. & N.Z. Soc. Cell Biol. **5**, (Abstr.).
5. Vize, P., Michalska, A.E., Robins, A.J., Seamark, R.F. and Wells, J.R.E. (1986) "Cloning of porcine growth hormone cDNA and its expression in transgenic mice". Proc. 8th Ann. Conf. on The Organization and Expression of the Genome, Lorne, Vic., Australia, (Abstr.).
6. Michalska, A.E., Vize, P., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1986) "Transgenic mice - growth hormone regulation by two porcine growth hormone gene constructs". Proc. Aust. Soc. Reprod. Biol. **18**: 12.
7. Michalska, A.E., Vize, P., Ashman, R.J., Stone, B.A., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1986) "Expression of porcine growth hormone cDNA in transgenic pigs". Proc. Aust. Soc. Reprod. Biol. **18**: 13.
8. Armstrong, D.T., Michalska, A.E., Ashman, R.J., Vize, P., Wells, J.R.E. and Seamark, R.F. (1986) "Gene transfer in goats". Proc. Aust. Soc. Reprod. Biol. **18**: 14.
9. Armstrong, D.T., Michalska, A.E., Ashman, R.J., Bessoudo, E., Seamark, R.F., Vize, P. and Wells, J.R.E. (1987) "Gene transfer in goats: methodologies and potential applications". Proc. IV International Conf. on Goats, Brasilia, Brazil.
10. Vize, P.D., Michalska, A.E., Ashman, R.J., Seamark, R.F. and Wells, J.R.E. (1987) "Improving growth in transgenic farm animals". Proc. EMBO Workshop on Germline Manipulation of Animals, Nethybridge, Scotland, Abstr. 42.

11. Michalska, A.E., Ashman, R.J., Stone, B.A. and Quinn, P. (1987) "Collection and transfer of single-cell pig embryos from slaughtered sows".
Aust. Vet. J. **64**: 90.
12. Michalska, A.E., Owens, P., Vize, P., Wells, J.R.E. and Seamark, R.F. (1987) "Expression of the porcine growth hormone gene in transgenic mice".
Proc. Endocr. Soc. Aust. **30**: 84.
13. Vize, P.D., Michalska, A.E., Ashman, R.J., Lloyd, B., Stone, B.A., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1988) "Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth".
J.Cell Sci. (in press).
14. Owens, P.C., Michalska A., Owens, J.A., Francis, G.L., McNamara, P., Conlon, M.A., Ballard, F.J., Wells, J.R.E. and Seamark, R.F. (1988). "Age-related differences in growth and metabolism of porcine growth hormone transgenic mice".
Proc. Endocr. Soc. Aust. **31**, (Abstr.).

ABBREVIATIONS.Chemicals

BSA	bovine serum albumin
CO ₂	carbon dioxide
DNA	deoxyribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
mRNA	messenger RNA
N ₂	nitrogen
O ₂	oxygen
RNA	ribonucleic acid
Tris	2-amino 2-hydroxymethyl propane-1, 3-diol

Hormones

GH (s)	growth hormone(s)
HCG	human chorionic gonadotrophin
IGF-1	insulin-like growth factor -1
IR IGF-1	immunoreactive IGF-1
IR pGH	immunoreactive porcine GH
PMSG	pregnant mares serum gonadotrophin

Symbols and units

bp	base pair
cm	centimetre(s)
Ci	curie
g	gram(s)
xg	unit of gravitational field
hr	hour(s)
IU	international units
l	litre(s)
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
mOsm	milliosmol(s)
ng	nanogram(s)

nm	nanometre(s)
°C	degrees centigrade (celsius)
pl	picolitre(s)
sec	second(s)
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)

Others

atm.	atmosphere
%	percent
<	less than
>	greater than
≥	greater than or equal to
CL	corpus luteum (corpora lutea)
cpm	counts per minute
e.g.	for example
et al.	et alia (and others)
Fig.	figure
HIHS	heat inactivated human serum
ID	inner diameter
OD	outer diameter
p (plural pp)	page(s)
PBS	phosphate buffered saline
psi	pounds per square inch
v/v	volume:volume
vs	versus
w/v	weight:volume