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Abstract: Since large animal transgenesis has been successfully attempted for the first time about 25 years ago, the technology has been applied in various lines of transgenic pigs. Nevertheless one of the concerns with the technology - animal welfare - has not been approached through systematic assessment and statements regarding the welfare of transgenic pigs have been based on anecdotal observations during early stages of transgenic programs.
The main aim of the present study was therefore to perform an extensive welfare assessment comparing heterozygous transgenic animals expressing GFP with wildtype animals along various stages of post natal development. The protocol used covered reproductory performance and behaviour in GFP and wildtype sows and general health and development, social behaviour, exploratory behaviour and emotionality in GFP and wildtype littermates from birth until an age of roughly four months.
The absence of significant differences between GFP and wildtype animals in the parameters observed suggests that the transgenic animals in question are unlikely to suffer from deleterious effects of transgene expression on their welfare and thus support existing anecdotal observations of pigs expressing GFP as healthy. Although the results are not surprising in the light of previous experience, they give a more solid fundament to the evaluation of GFP expression as being relatively non-invasive in pigs. The present study may furthermore serve as starting point for researchers aiming at a systematic characterization of welfare relevant effects in the line of transgenic pigs they are working with.

Welfare assessment in transgenic pigs expressing green flurescent protein (GFP)

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Abstract

Since large animal transgenesis has been successfully attempted for the first time about 25 years ago, the technology has been applied in various lines of transgenic pigs. Nevertheless one of the concerns with the technology - animal welfare - has not been approached through systematic assessment and statements regarding the welfare of transgenic pigs have been based on anecdotal observations during early stages of transgenic programs.

The main aim of the present study was therefore to perform an extensive welfare assessment comparing heterozygous transgenic animals expressing GFP with wildtype animals along various stages of post natal development. The protocol used covered reproductory performance and behaviour in GFP and wildtype sows and general health and development, social behaviour, exploratory behaviour and emotionality in GFP and wildtype littermates from birth until an age of roughly four months.

The absence of significant differences between GFP and wildtype animals in the parameters observed suggests that the transgenic animals in question are unlikely to suffer from deleterious effects of transgene expression on their welfare and thus support existing anecdotal observations of pigs expressing GFP as healthy. Although the results are not surprising in the light of previous experience, they give a more solid fundament to the evaluation of GFP expression as being relatively non-invasive in pigs. The present study may furthermore serve as starting point for researchers aiming at a systematic characterization of welfare relevant effects in the line of transgenic pigs they are working with.

Keywords: GFP, transgenic pig, welfare, behaviour

Introduction

Since the first successful creation of transgenic large animals about 25 years ago, various lines of transgenic pigs have been developed for applications ranging from potential models for human diseases to the improvement of production parameters (Laible 2009; Niemann et al. 2005; Clark and Whitelaw 2003). Possible effects of genetic modification on the welfare of the animals involved have been a concern, both from a general moral and public opinion point of view (Einsiedel 2005; Van Reenen 2007; Olsson and Sandoe 2003; Lassen et al. 2005; Kaiser 2005) and because early research had generated animals with some substantial health and welfare problems (reproduction, susceptibility to stress and lameness, Clark and Whitelaw 2003). However, although potential factors that may affect transgenic large animal welfare have been identified and recommendations regarding welfare assessment within different stages of a transgenic program have been published (Van Reenen et al. 2001; Van Reenen 2007) there is still a lack of studies on transgenic large animals in general and transgenic pigs in particular where a comprehensive and systematic welfare assessment was performed in a number of animals sufficiently high for statistical analysis.

The main aim of the present study was therefore to perform such a study, characterizing the welfare of transgenic pigs expressing Green Fluorescent Protein (GFP), which is commonly used as a reporter in proof of principle studies testing (novel) gene delivery constructs (e.g.Hofmann et al. 2003; Li et al. 2009; Whitelaw et al. 2004) where it allows to visualize gene expression real time and in vivo. It has been considered to be ´non-invasive´ (Hadjantonakis and Nagy 2001) and transgenic pigs expressing GFP have been described as normal (Li et al. 2009; Brunetti et al. 2008; Kurome et al. 2006; Webster et al. 2005) and healthy (Cabot et al. 2001) before. However, these assumptions were based on anecdotal observations in relatively few animals. While this kind of information can be valuable and necessary in initial stages of a large animal transgenic program, systematic welfare assessment using detailed protocols is needed to adequately describe the phenotype and welfare of transgenic large animals once the number of animals involved is increased (Van Reenen 2007).

As there have been no reports of specific adverse effects on animals expressing GFP in the present study a broad approach was applied with a protocol constructed in the light of recommendations regarding welfare assessment and phenotyping of genetically modified mice (Crawley 2007; Rogers et al. 1997; van der Meer et al. 2001) and farmed pigs (Webster 2005; Velarde and Geers 2007; Smulders et al. 2006). Heterozygous transgenic pigs expressing GFP (GFP pigs) were compared to wildtype (wt pigs) littermates (Supplementary Fig. 1) regarding health and development, social behaviour, exploratory behaviour and emotionality. Additionally heterozygous GFP sows (primiparous) were compared with wt sows regarding reproductory success and behavioural time budget.

Animals, Material and Methods

Animals

Data collection took place at The Roslin Institute (UK) large animal unit between February (birth of first piglets) and June 2008 (last weighing procedure). Details on the generation of the founder animals using lentiviral vectors are described in Whitelaw et al 2004. In short, founder animals were created by using an equine infectious anaemia virus derived vector resulting in animals with high levels of GFP expression in various organs and the exocrine system (Whitelaw et al. 2004; Vasey et al. 2009). The underlying genotype of the animals was a 3 - way cross between Large White, Landrace and Duroc. In the present study, F3 gilts were impregnated at 6.5 months of age by artificial insemination in 2 batches about 1 month apart. Wt females (n=8) were inseminated with semen from one heterozygous tg boar and GF females (n=10) with semen from one wt boar which resulted in litters with roughly 50% heterozygous tg and 50% wt piglets. The gilts were housed in mixed (genotype) groups of 5 to 8 animals and moved to the farrowing shed 5 days prior to the expected day of parturition. The newborn piglets (F4) were marked individually on day 1 (=day of birth) or 2 by ear notching. Genotyping was performed with FHS/F-00 goggles for fluorescent protein visualization (BLS Budapest, Hungary, Supplementary Fig. 1). Additionally ear notches and tail clips were stored as samples for later confirmation of the genotype via PCR.

The 18 females included in the study gave birth to 209 piglets in total, 183 of which were born alive. Litter size was reduced, if necessary, to a maximum of 12 piglets per sow to ensure the availability of sufficient functional teats. At weaning at 28 +-1 days of age 80 piglets (40 GFP, 40 wt) were moved from the farrowing unit to the adjacent housing unit and randomly allocated to same sex groups of 4 wt and 4 GFP animals each. 39 animals were kept to an age of roughly 17 weeks. From birth to the age of 119 days the offspring was subjected to general health and development monitoring (´scans´) and a battery of behavioural tests (time line Fig. 1).

Housing and procedures

Prior to weaning, the animals were housed litter wise with their mother in Solari farrowing units (180x500cm) with ambient temperatures ranging from 14 to 17°C. Additional heat was supplied via a heat lamp above the creep area in each pen and during February, March and April using two butane fed gas lamps for the farrowing unit. The pens were mucked out and fresh straw was provided daily. Food (Supreme Lactation Pellets, ABN Ltd. Peterborough UK) was given to the sows twice daily (at 8am and 4pm) according to a predefined program with increasing amount along lactation, water being provided ad lib in a bowl drinker. Additionally, a low quantity (1 fist full) of pig starter feed (Scotlean Silver Pellets, ABN Ltd. Peterborough UK) was allocated on the floor of the creep area for habituation to solid food.

Pens for weaned animals (310 x 400cm) in the same shed as the farrowing unit were mucked out twice per week and fresh straw was provided if necessary. Temperature was similar to the farrowing unit, food and water (nipple drinker) was provided ad lib.

On the first day, piglets were routinely given an iron injection (1 ml Gleptosil, Alstoe Ltd. York UK). Oral litter wise treatment against diarrhoea (Spectam Scour Halt, AmTech Ltd.) was performed when one or more animals in a litter showed signs of diarrhoea (e.g. faecal streaks). Animals with lesions were treated by intramuscular injection of Pen&Strep (Norbrook, UK) and a disinfectant spray was applied to open wounds.

Euthanasia was performed according to regulations in the Animals (Scientific Procedures) Act 1986 in the rare case of an animal suffering from a deteriorated health status (illness, lesions) and/or pain with no prospect of recovery. Animals were also euthanized when they were neither needed for the present study nor within other studies at the Roslin Institute or associated institutions.

Reproduction and behaviour of sows

Parameters of reproductory performance of the sows were recorded as gestation length, number of piglets born in total, born alive, stillborn/ died at birth and the number of piglets lost from birth to weaning. The litters were filmed and videos recorded from 1.00pm to 4.00pm on two consecutive days around 14 days of piglets´ age to observe sow behaviour regarding time budget. Behaviour was analysed using instantaneous sampling with sample intervals of 180 seconds (Martin and Bateson 1993). The ethogram (Supplementary Table 1) for this purpose was adapted from (Munsterhjelm et al. 2008). If the sow was in the process of lying down at a sample point, the behaviour was recorded as the lying position assumed immediately after lying down, if at the sample point the sow was getting up, the subsequent behaviour was recorded.

Welfare assessment before and after weaning in offspring – general health, development and reactivity

Piglets were closely monitored individually weekly from birth (Day 1) until weaning (age 28 +/- 1 days post partum) and 35 +/- 1 days post partum regarding development, reflexes, general health and appearance (parameters see Table 1) using score sheets. Parameters were screened for at the time when they are most likely to occur (i.e. for example condition of navel early in development, rectal prolapse later). In procedures before weaning all piglets of a litter were removed from their pen and brought to the entrance area of the farrowing shed where they were weighed and individually screened regarding general appearance parameters. Rectal temperature was measured as close after birth as possible using a mercury thermometer. One week after weaning, the animals were individually removed from their pen and screened regarding general health and appearance parameters again. Suspicious clinical condition outside scheduled assessment days were detected and if necessary treated by care takers and/or researchers.

A back test was performed once with each animal at Day 7 adapted from van der Kooij et al to assess the animals´ reactivity and coping behaviour (van der Kooij et al. 2002). In brief the animals were placed dorsally on a horizontal rubber mat and kept in that position by the experimenter who placed his left hand loosely upon the head/neck of the animal and holding the back legs loosely with the right hand. Events of wriggling (´escape events´) were counted during 60 seconds.

Teat order, lesion assessment, resident/intruder test – social behaviour

A teat order study was performed 14 days of age as by then the teat order has been established and teat fidelity is high (Puppe and Tuchscherer 1999; Litten et al. 2003). The piglets were individually numbered with a non toxic marker pen on forehead (Supplementary Fig. 1 b), flanks and back and on the following morning (9.00am to 1.00pm) 2 suckling events were observed for each litter. When milk was visibly ingested the teat row number for each piglet on each occasion was recorded, 1 being in the most anterior (i.e. closest to the sow´s head), 7 the most posterior row. For analysis of udder region fidelity the udder was divided in 3 regions anterior (most anterior 2 teat rows), middle (middle 3 teat rows) and posterior (most posterior teat rows).

Lesions were counted on the left lateral side of the piglets´ body just before weaning at 28 days and one week after weaning in the following regions: face/head, backside of the ears, shoulder, front legs, flanks and tail (Supplementary Fig. 2). For practical reasons (impossibility to distinguish many overlapping lesions) 5 or more lesions were recorded in one category as ´>5´. For statistical analysis, the counts were divided into 3 categories: Low lesion number (0 and 1 lesion), medium number (2, 3, or 4 lesions) and high number of lesions (5 or more). Additionally the severity of lesions on tail and ears was recorded one week post weaning using a scoring system according to Smulders et al (Smulders et al. 2006). It was assessed with scores 1 (without any lesions), 2 (only superficial scratches) or 3 (deep scratches with fresh blood or crusted blood). Lesions with a severity score of 4 (severe lesions where parts of the tail or ears were missing) as described by Smulders et al were never observed in the present study and therefore omitted.

A Resident/Intruder test was performed following a protocol adapted from D’Eath (D'Eath 2005). Prior to testing, residents were habituated to the 1.80 x 4.00 m test arena within the homepen (= pen excluding the dunging area) on two occasions 2 and 1 days before the test respectively. For this purpose, the animals were moved into the arena in randomly selected penmate pairs and allowed to explore freely for 5 min.

In the test, 38 subjects of batch 1 acted as ‘residents’ (GFP residents: n=20, wt residents: n=18) and 38 sex and genotype matched unfamiliar ‘intruder’ pigs from batch 2 were used. Intruder pigs were around 40% of the resident’s weight. Testing was performed pen wise in random order. A single ‘resident’ pig was moved into the test arena and the intruder introduced. By giving the resident two advantages, home pen residency and size, the resident has the initiative as to whether or not to attack. An attack was defined as a sudden attempt to deliver a rapid series of five or more head knocks or bites to the other pig. The test was stopped and the pigs separated when an attack occurred or after a cut off time of 5 min. Each resident was tested on two occasions on different days, using different intruders each time. Each intruder was used twice in total, and only once per day. The animals were compared regarding the latency to attack and the latency to establish the first snout contact by the ´resident´ with any part of the intruder’s body. Latency to attack was defined as the time from the first contact by the residents’ snout with any part of the intruder until an attack occurred. Pigs that did not perform an attack were excluded from statistical analysis of attack latency.

Open field, Novel object and Human approach test – Exploratory activity and emotionality

Around 119 days of age 39 animals (19 wt, 20 GFP) were individually tested regarding general activity in and their reaction to an unfamiliar environment (Open Field tests – OF1, OF2), an unfamiliar object (Novel Object Test – NOT) and an unfamiliar human (Human Approach Test – HAT) following adapted protocols from Pearce and Paterson (Pearce and Paterson 1993). At the end of the tests, the number of faecal boli was counted and the arena dry cleaned before testing the next animal. An adapted post weaning pen divided into 6 areas was used as arena (Supplementary Fig. 3). The lines indicating the areas and a distance of approx 0.5m from the novel object and human respectively had been painted on the floor 1 week before testing. NOT and HAT were performed in random order on 2 consecutive days when the animals were around 17 weeks old. Open field, NOT and HAT tests were videotaped and behaviours were recorded continuously.

All video analysis was performed using The Observer® Software (Noldus Netherlands).
On the first day of testing the animals were manoeuvred individually into the arena and left to explore freely for 5 minutes (OF1). Then a metal bucket on a string was introduced in the corner opposite the entrance (Supplementary Fig. 3) in a way that it was touching the floor but could not be moved away by the pigs. After allowing the animal to explore the object for 3 minutes (NOT) the test was ended and the pig moved back to the home pen.
On the second day the open field test was repeated (OF2) and a human previously unfamiliar to the animals entered the pen and stood in the same corner used in the NOT. The human was dressed in overall and rubber boots as used by farm staff and experimenter and was allowed to gently push away the pig when it bit (Chaloupkova et al. 2007).
Analogous to Pearce and Paterson the following parameters were recorded: 1) the latency to cross the line within 0.5 m of the novel object/human with the nose; 2) the latency to first physically interact (sniff, touch, manipulate) the novel object/human; 3) the total time spent within 0.5 m of the novel object/human with the nose; 4) the total time spent interacting with the novel object/human; 5) the number of interactions with the novel object/human.

Statistical Analysis and software used

To determine significant differences between GFP and wildtype animals, statistical analysis was performed using the SPSS 16.0 software package with a pre set level of significance < 0.05. As most data did not meet the necessary assumptions for parametric tests, non-parametric tests were applied (Mann Whitney U or Chi square test).
To test the difference of weight development between genotypes first a linear model using a generalized estimating equation with weight as a function of time (pig's age at the measurement) was fitted for each sex separately. This kind of equation takes into account the repeated measures and is not sensitive to missing data. The interaction between time and genotype was tested to determine if the weight development was different between GFP and wildtype animals. To improve fit, another model with a quadratic term for time was applied. Again the

interactions between time variables (time and time_squared) and genotype were tested.

A note on the ´blindness´ of the observer towards the animal´s genotype

Under natural light GFP expression was visible in the snout and trotters of the animals, rather faintly in newborn animals but with increasing intensity along development (Supplementary Fig. 1). During direct observation/screening from birth to weaning and during the Resident/Intruder test, the observer was therefore inevitably aware of the genotype of the animals. The observers can be considered blind to the genotype however in data based on video analysis (Open field, Novel object, Human approach, Sow behaviour) as the animals were given random ID´s for analysis and it was impossible to discern the genotype of the animals from the black/white video.

Results

Reproduction and behaviour of sows

No statistically significant difference between GFP and wiltype sows regarding reproductive performance and litter loss was found: neither gestation length, nor the number of piglets born in total, alive or stillborn or the number of piglets lost from birth to weaning differed between gilts of the two genotypes (Table 1). Out of 13 piglets that had to be put down from birth to weaning for reasons common in pig production (e.g. broken legs, not healing skin wounds), 5 were wt and 6 GFP while 2 were of unidentified genotype; 5 GFP and 3 wt females lost no piglets from birth to weaning. Behaviour of sows did not vary significantly between genotypes but showed a high variability within genotypes. Sows spent roughly 50% of the observed time lying inactively (48.5% and 53.2% of sample points in GFP and wt sows respectively) followed by suckling (23.3% and 21.0% in GFP and wt sows respectively) and exploring either the pen and its fittings or the straw (total exploring 11.3% and 13.0% in GFP and wt sows respectively; Supplementary Table 2).

Welfare assessment before and after weaning in offspring – general health, development and reactivity

When analysed by application of a linear as well as a quadratic model, GFP and wildtype offspring of both sexes showed no statistically significant difference in weight development along the time of the study. (p values >0.05; Fig. 2 and Supplementary Table 3). Early development, general health and appearance of offspring were similar in GFP and wt animals. Neonatal rectal temperature did not differ significantly, being 37.1 and 37.2 ° C (p=0.273) in GFP and wt animals respectively. Similarly, none of the other screening parameters assessed from birth to weaning and 1 week post weaning revealed significant differences between GFP and wt piglets (Table 2). During the 60 seconds backtest at 7 days of age, both GFP and wt animals struggled around 4 times (p=0.348, Table 2) with a range of 2 to 7 and 2 to 8 struggling bouts in GFP and wt piglets respectively.

Teat order, lesion assessment, resident/intruder test – social behaviour

The use of teat pairs and udder regions for suckling did not differ between wildtype and GFP piglets (p=0.808 and p=0.661 for suckling 1 and suckling 2 respectively; Chi square test) and both genotypes showed high teat pair and udder region fidelity: 79% and 86% of GFP and wt piglets suckled teats of the same row (p=0.303) and 92% of the animals in both gentoypes suckled teats in the same udder region (anterior, middle or posterior, p>0.999) in the two milk let downs recorded.

When comparing the distribution of piglets in the lesion count categories no significant difference was found between GFP and wt animals in any of the body areas assessed. Only the distribution of piglets in lesion count categories of the shoulder area at weaning approached statistical significance (p=0.083, Supplementary Table 5). No significant difference between the two genotypes was found in the distribution of animals in the lesion severity categories one week after weaning. At that time, a large proportion of animals (≥ 88%) in both genotypes had deep lesions with fresh or crusted blood (category 3) on body and ears (Supplementary Table 6) indicating increased fighting after mixing previously unfamiliar animals.

In the resident/Intruder test GFP and wt resident pigs weighed on average 40.6 and 42.6 kg respectively at the time of testing. Resident/Intruder test data was not normally distributed (exploratory data analysis and tests of normality, data not shown) and therefore were analysed using non parametric tests (Table 3). Latency to establish snout contact with any part of the intruders' body was around 6 seconds (median) in the first and dropped to around 2 seconds in the second test session with no difference between genotypes. Latency to attack did not differ significantly between GFP and wt animals with values around 35 seconds (median) for attack latency in both test sessions (Table 3). During the first test session 3 GFP and 2 wt animals did not attack and 2 GFP and 4 wt animals (including the 2 who had not attacked in the first session) did not attack during the second test. No intruder attack was observed.

Open field, Novel object and Human approach test – Exploratory activity and emotionality

There was no significant difference in the level of overall activity as measured by the number of total zone entries in the two open field tests (OF1 and OF2) between genotypes. GFP animals had a mean of 39.7 and 30.2, wt animals 41.1 and 33.0 zone entries in the OF1 test and OF2 test respectively (p values 0.764 and 0.993 respectively, Mann Whitney U test). The total time spent in each zone did not differ between GFP and wt animals (Fig. 3) and overall, no significant differences between genotypes in the behaviours performed during OF1 and OF2 were detected (Table 1). Animals of both genotypes showed roughly the same priorities in terms of time spent on different behaviours: exploring (either while walking or standing) > standing inactively > walking without other activity > manipulating doors. Most time was spent exploring the arena in both tests, either while walking or motionless with a distinct decline from OF1 to OF2 in both genotypes especially in exploration during walking (total exploring activity around 78% in GFP and 81% in wt animals in OF1 and 53% in GFP and 59% in wt animals in OF2 respectively). On the other hand, duration of inactive standing (12% in GFP and 11% in wt animals in OF1 and 30% in GFP and 24% in wt animals in OF2 respectively) as well as walking without other activity (6% in GFP and 3% in wt animals in OF1 and 11% in GFP and 10% in wt animals in OF2 respectively) showed marked increases from OF 1 to OF 2.

No statistically significant difference was found between animals from the two genotypes in their reaction to an unfamiliar object in the 180s NO test, however variability within groups was high (Table 3). Both GFP and wt animals quickly entered the 0.5 m area around the novel object (median latency 2.8s and 3.4s after the introduction of the object respectively; p=0.679) and established contact with the novel object few seconds later (median latency 6.3s and 8.2s respectively; p=0.297). Animals in both groups spent more about one fifth of the time interacting with the object (median 28.2% and 23.3% respectively; p=0.822) and spent almost half of the time in zone 1 where the novel object was introduced (Fig. 3; median 46.5 and 46.9% in GFP and wt animals respectively; p=0.556) GFP and wildtype animals reacted in a similar manner to an unfamiliar human (Table 3). After almost immediate entry into the the zone around 0.5m within the human, the median time to establish snout contact was 3.5s and 3.4s for GFP and wildtype animals respectively (p=0.877). GFP and wildtype animals spent 12.8% and 11.4% of the test respectively interacting with the human (p=0.306) and most time was spent in zone 1 where the unfamiliar human was standing during the test (Table 3; median 37.1 and 27.0 % of the test respectively; p=0.089).

Discussion

To our knowledge, this was the first time a comprehensive welfare assessment was performed in genetically modified pigs. F3 GFP and wt sows were compared regarding reproductory performance and behaviour. Their offspring, GFP and wt F4 full siblings were monitored regarding general health and appearance, pre weaning mortality, weight development and emotional, exploratory and social behaviour from birth to the age of approximately 17 weeks. No statistically signifiant differences were found between GFP and wildtype animals in the parameters measured.

The choice of parameters and the number of animals was adapted to the stage of the transgenic program, i.e. as no adverse effects of GFP had been observed in the founder generation a broad approach was attempted and an elevated number of animals was monitored to be able to detect possible small, but biologically relevant adverse effects of transgene expression. The monitoring period represents the developmental phase from birth to puberty and early adulthood in offspring and the reproductive phase in sows, and transgenic and wildtype full siblings were compared to avoid litter effects (Van Reenen 2007). Although there are parameters that were not observed due to technical constraints and for reasons of feasibility in the present study, the welfare assessment was comprehensive in that it comprises a cross-section of welfare relevant domains covering elements of biological functioning (reproductory performance, offspring [weight] development, lesion assessment) behaviour and emotional state of the animals involved (Duncan 2005).

Based on the parameters observed in the present study, GFP expression per se does not seem to influence the welfare and behavioural phenotype in the pigs involved: Indicators of reproductory performance are within range of data typical for the species (van Dijk et al. 2005) in both genotypes and the behaviour of the sows seems to be unaltered by GFP expression. Similarly, the results of the present study indicate that the general health and development of offspring expressing GFP is not modified.

Despite of the lack of statistically significant differences between genotypes the parameters observed, the results coincide with common patterns of pig development and behaviour. Animals of both genotypes in the present study showed a high teat pair and udder region fidelity when observed, indicating that a stable teat order had been established by the age of 2 weeks. Udder region use has been linked to weight gain and dominance in piglets with animals suckling posterior teats tending to grow slower and being less dominant (Puppe and Tuchscherer 1999; Litten et al. 2003). In the present study, GFP piglets do not appear to be disadvantaged in the access to the anterior udder region. Animals across genotypes showed typical features of physical development and general health and appearance, for example concerning carpal lesions (Zoric et al. 2008): competition for teats is fierce between piglets until a stable teat order is established. Therefore carpal abrasions caused by friction with the pen floor when trying to access the udder were frequent during the first week (data not shown) and declined during the following weeks until at weaning when no lesions were present.

Offspring of both genotypes showed similar species specific behaviour both in relative (i.e. priorities of behaviours) and absolute terms (i.e. no significant difference between values) when tested within basic behavioural tests. The novel object and human approach tests indicate a similarly high interest in an unfamiliar object and an unfamiliar human and revealed no difference in the level of anxiety in animals of both genotypes. This is indicated by the elevated amount of time spent in the zone containing the novel object and the human respectively and in the high percentage of time spent in interaction despite of the novelty of the situation.

The overall results confirm the statements on the absence of differences between wildtype pigs and pigs expressing GFP in previous studies (Li et al. 2009; Brunetti et al. 2008; Kurome et al. 2006; Webster et al. 2005; Cabot et al. 2001) and experience at the Roslin Institute. The comparatively high number of animals and the broadness of parameters observed, however, put conclusions on the absence of detrimental effects of the transgene expression in question on the welfare of the animals involved on a more robust and verifiable basis.

Conclusions

In summary, GFP expression in itself as induced by the method used (Whitelaw et al. 2004) does not seem to have unexpected or undesirable effects on the pigs involved and it is unlikely that the welfare of the transgenic animals is compromised. This result does not come as a surprise in the light of previous experience on pigs expressing GFP at The Roslin Institute and other institutions. However, the present study may serve as an example and starting point for researchers aiming to describe the welfare impact and phenotypic consequences of the genetic modification they are working with.

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Figure captions

Fig. 1 Time line of welfare monitoring and behavioural testing in GFP and wt offspring. Scan – general health and appearance scan, BT – back test, TO – teat order, LA – lesion assessment, RI – resident/intruder test, OF/NO – open field/novel object test, OF/HA – open field human approach test

Fig 2 Weight development of female and male GFP (dashed line) and wt (continuous line) offspring aged 1 to 119 days; Values represent mean body weight and error bars represent SD

Fig. 3 Time spent in the 6 zones of the testing arena (see Supplementary Fig. 3) in the 5 minute Open field tests preceding the NO test (1) and the HA test (2) and during the 3 minute NO test (3) and HA test (4). Data is represented as the median percentage of time the animals of the respective genotype (GFP animals – white bars, wildtype animals – black bars) spent in each zone of the arena during the test session. Random zone use would mean 16.7% of the time spent in each zone

Abstract

Since large animal transgenesis has been successfully attempted for the first time about 25 years ago, the technology has been applied in various lines of transgenic pigs. Nevertheless one of the concerns with the technology - animal welfare - has not been approached through systematic assessment and statements regarding the welfare of transgenic pigs have been based on anecdotal observations during early stages of transgenic programs.

The main aim of the present study was therefore to perform an extensive welfare assessment comparing heterozygous transgenic animals expressing GFP with wildtype animals along various stages of post natal development. The protocol used covered reproductory performance and behaviour in GFP and wildtype sows and general health and development, social behaviour, exploratory behaviour and emotionality in GFP and wildtype littermates from birth until an age of roughly four months.

The absence of significant differences between GFP and wildtype animals in the parameters observed suggests that the transgenic animals in question are unlikely to suffer from deleterious effects of transgene expression on their welfare and thus support existing anecdotal observations of pigs expressing GFP as healthy. Although the results are not surprising in the light of previous experience, they give a more solid fundament to the evaluation of GFP expression as being relatively non-invasive in pigs. The present study may furthermore serve as starting point for researchers aiming at a systematic characterization of welfare relevant effects in the line of transgenic pigs they are working with.

Table

Table 1 Reproduction performance in GFP and wt sows

	GFP sows (n = 10)		Wt sows (n = 8)		P value
	Mean (Range)	SD	Mean (Range)	SD	Mann-Whitney U
Gestation length in days	113.9 (111 – 116)	1.5	114.6 (113 – 116)	1.1	0.423
Number of total born piglets	11.8 (2 – 17)	4.1	11.4 (7 – 13)	2.0	0.573
Number of live born piglets	10.8 (2 – 16)	3.8	9.4 (5 – 13)	2.4	0.173
Number of piglets stillborn/died at birth	1.0 (0 – 3)	1.2	2.0 (0 – 4)	1.2	0.122
Number of piglets lost from birth to weaning	0.7 (0 – 3)	0.9	0.8 (0 – 2)	0.7	0.696

Table

Table 2 Physical development and general health and appearance screened weekly before weaning at 28 days of age and one week after weaning

Parameter/Age	1	7	14	21	28	35
Signs of diarrhoea	0.183**	0.503**	0.630**	0.085**	constant	constant
Signs of dehydration	0.444**	0.444**	constant	>0.999**	constant	constant
Condition of skin	>0.999**	0.795**	0.444**	0.131**	>0.999**	constant
Sneezing/coughing/nasal discharge	0.447**	>0.999**	constant	>0.999**	constant	constant
Activity	0.444**	constant	constant	constant	constant	constant
Rectal temperature	0.273*	na	na	na	na	na
Number of nipples	0.550*	na	na	na	na	na
Congenital tremor	constant	na	na	na	na	na
Splay leg	constant	na	na	na	na	na
Condition of eyes	constant	na	na	na	na	na
Cleft palate/jaw position/harelip	constant	na	na	na	na	na
Hernia	constant	na	na	na	na	na
Atresia ani (blind anus)	constant	na	na	na	na	na
Palpebral reflex	constant	na	na	na	na	na
Condition of teeth	constant	na	na	na	na	na
Condition of umbilicus/navel bleeding	0.585**	>0.999**	na	na	na	na
Vocalisation during handling	0.299**	>0.999**	na	na	na	na
Facial lesions	0.667**	0.466**	0.259**	0.202**	na	na
Carpal lesions	>0.999**	0.746**	>0.999**	0.637**	na	na
Body lesions	0.423**	0.155**	0.853**	0.732**	na	na
Vomiting	na	constant	constant	constant	constant	na
Lameness	na	0.196**	0.193**	>0.999**	>0.999**	constant
Swollen joints	na	0.086**	0.441**	0.255**	>0.999**	
Condition of claws	na	0.579**	constant	0.585**	>0.999**	constant
Condition of fur	na	constant	constant	>0.999**	constant	constant
Backtest	na	0.348*	na	na	na	na
Condition of eyes	na	na	0.441**	0.135**	>0.999**	constant
Condition of ears	na	na	na	na	constant	na
Condition of tail	na	na	na	na	>0.999**	na
Discharge anogenital region	na	na	na	na	constant	na
Rectal prolapse	na	na	na	na	constant	na

The value given for a certain parameter at a certain age is the p-value for the statistical difference between GFP and wildtype offspring. * Mann Whitney U test; ** Fisher´s exact test, 2 – sided; constant: no animals showed conspicuous condition in both genotypes; na: parameter not assessed at given age

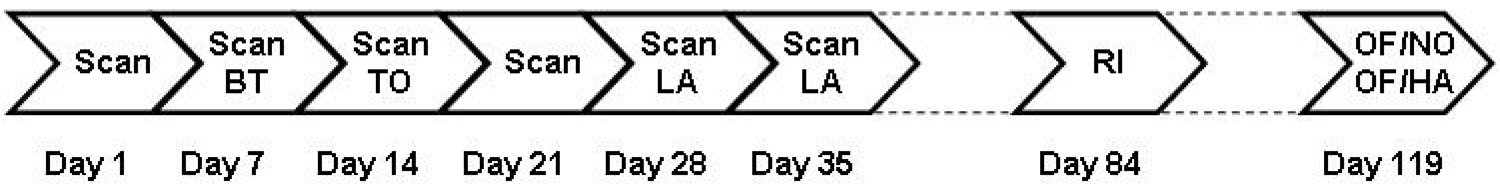
Table

Table 3 Results of behavioural tests

Test	Parameter	Unit	GFP animals	SD	Wt animals	SD	P value Mann Whitney U
R/I 1	Snout contact latency	s	11.0	17.1	10.9	13.9	0.744
	Attack latency	s	48.6	42.8	51.2	40.3	0.650
R/I 2	Snout contact latency	s	2.8	1.6	2.2	1.2	0.240
	Attack latency	s	51.1	56.6	35.2	24.3	0.992
OF prior to NO	Time spent Standing	%	11.7	7.6	10.5	8.4	0.461
	Time spent Locomotion	%	5.6	5.5	3.4	3.4	0.254
	Time spent manipulating door	%	4.2	3.0	5.2	7.2	0.839
	Time spent investigating while walking	%	55.1	13.0	57.5	10.9	0.443
	Time spent investigating while standing	%	23.5	5.4	23.3	7.0	0.988
OF prior to HA	Time spent Standing	%	29.7	17.5	23.8	15.3	0.358
	Time spent Locomotion	%	11.0	8.6	9.9	5.5	0.817
	Time spent manipulating door zone 1	%	0.8	1.3	1.5	1.9	0.303
	Time spent manipulating door zone 2	%	5.7	9.5	5.4	5.1	0.443
	Time spent investigating while walking	%	27.7	20.0	29.1	12.2	0.340
	Time spent investigating while standing	%	25.1	14.1	30.2	10.7	0.094
NO	Latency to enter within 0.5m of novel object	s	4.0	3.3	10.5	21.3	0.679
	Latency to interact with novel object	s	8.0	4.6	15.1	21.3	0.297
	Time spent within 0.5m if novel object	%	12.2	6.2	11.1	4.5	0.988
	Time spent interacting within novel object	%	33.1	24.1	37.8	32.0	0.822
	Number of interactions with novel object	n	6.0		5.0		0.733
HA	Latency to enter within 0.5m of human	s	2.6	4.4	11.0	25.4	0.781
	Latency to interact with human	s	8.9	18.4	12.5	25.6	0.877
	Time spent within 0.5m of human	%	11.0	6.8	9.3	5.7	0.314
	Time spent interacting with human	%	16.7	11.2	13.1	9.3	0.306
	Number of interactions with human	n	6.5		6.0		0.244

Results are represented as mean and standard deviation (SD) for continuous and median for count data. Values in percent indicate the proportion of total test time spent performing the respective behaviour . R/I = Resident/Intruder test; OF = Open field test; NO = Novel object test; HA = Human approach test

Line figure


Scan
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Day 1
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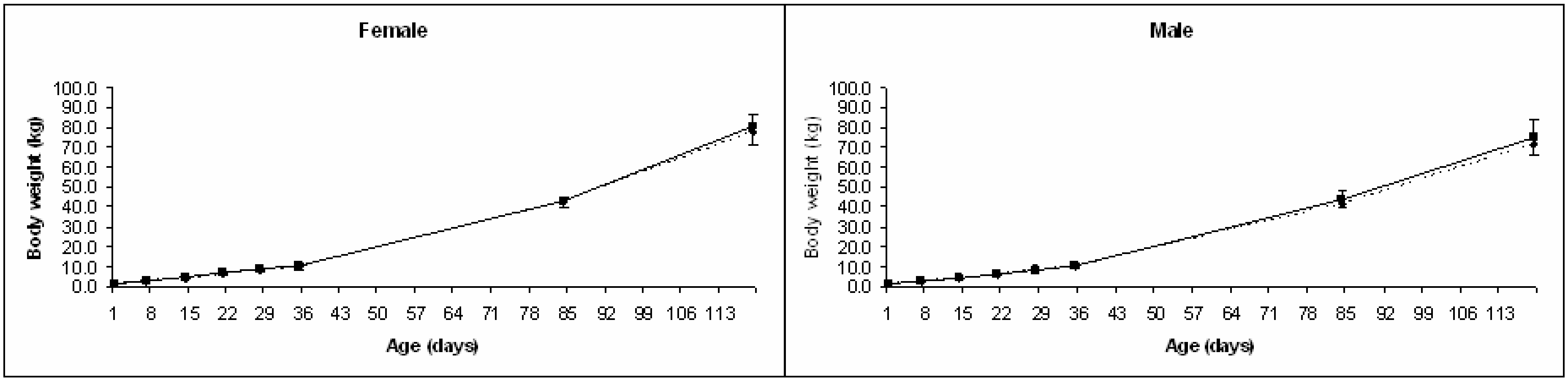
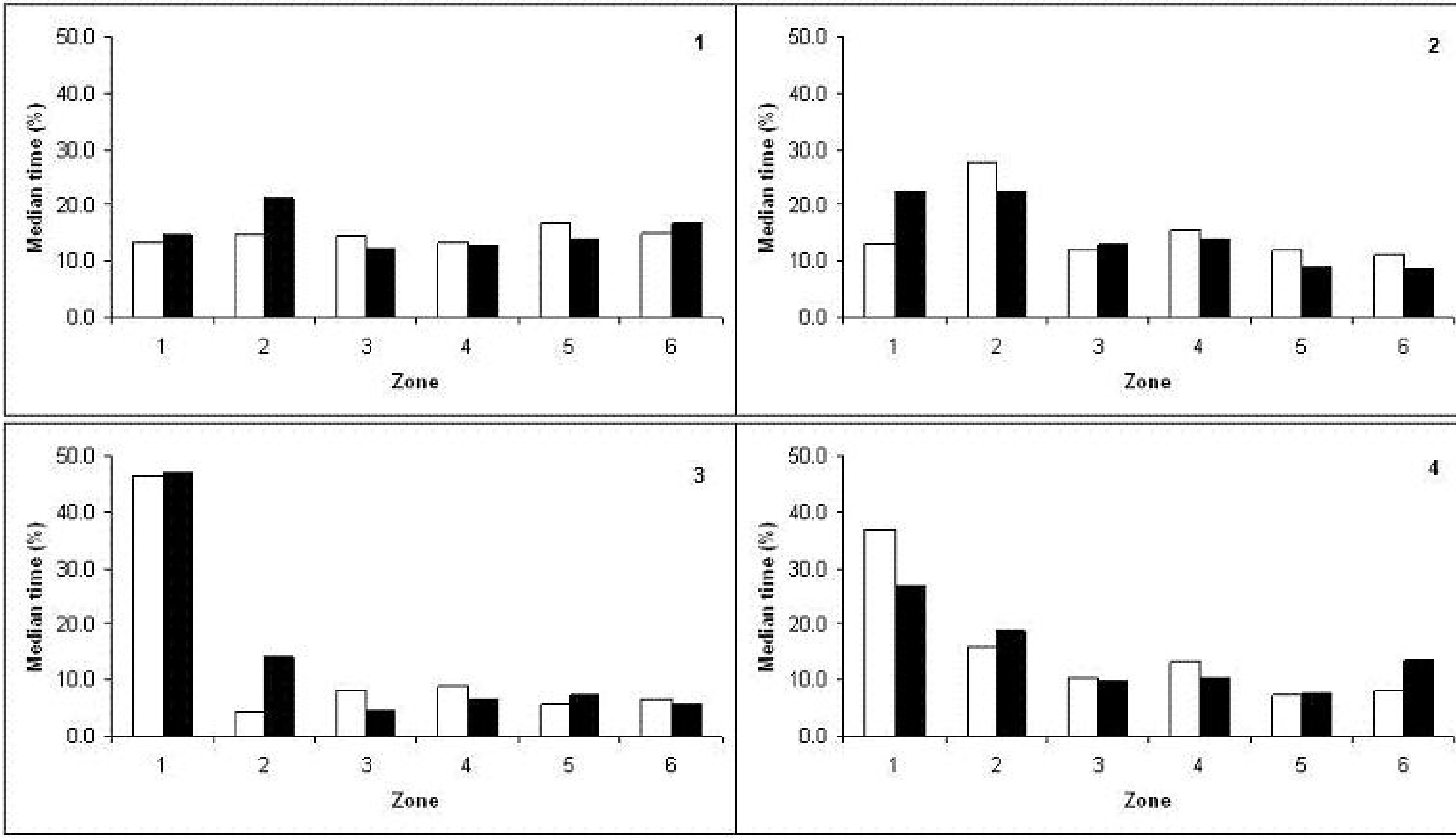
Day 35
Day 84
Day 119

Line figure



Female
Male
Body weight (kg)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106 113
Age (days)

Line figure



Attachment to manuscript

Supplementary figures

Welfare assessment in transgenic pigs expressing green flurescent protein (GFP)

Transgenic Research

Reinhard C. Huber[1,3,5], Liliana Remuge[1], Ailsa Carlisle[2], Simon Lillico[2], Peter Sandøe[3], Dorte B. Sørensen[4], C. Bruce A. Whitelaw[2,] I. Anna S. Olsson[1,3]

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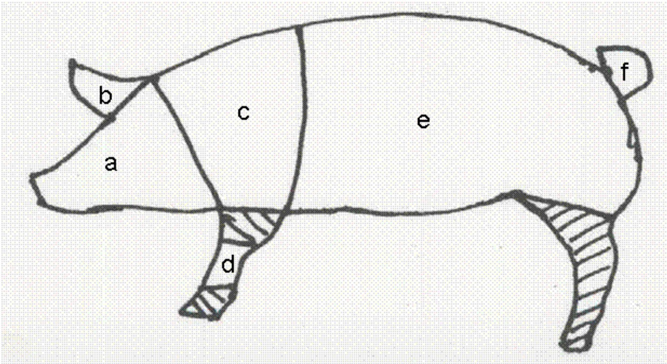
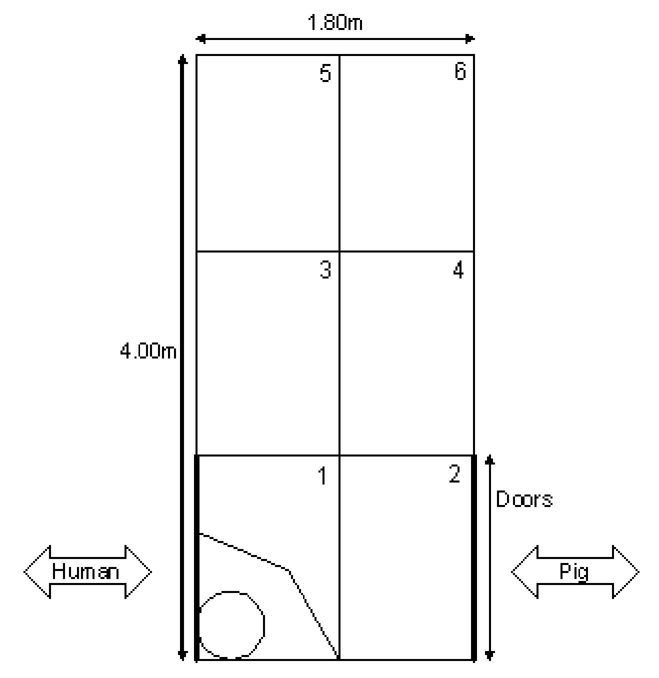
Supplementary Fig. 1 a: Wt and GFP littermates as seen through GFP expression visualization equipment. b below: A mixed litter marked for teat order observation. Piglets numbered 4, 1 and 8 are GFP, 9, 2 and 6 wt

Supplementary Fig. 2 Body region scheme used for lesion assessment: a – head/face, b – ear, c – shoulder, d – carpus, e – flank, f – tail; Lesions occurring in the striped areas were not recorded



Supplementary Fig. 3 Arena for Open Field/Novel Object and Open Field/Human Approach tests. The position of the novel object and the unfamiliar human is indicated by the circle. The arrows show entry and exit of the pig tested/human, numbers indicate the zones used for analysis of activity in the OF, NO and HA tests



Supplementary Tables

Welfare assessment in transgenic pigs expressing green flurescent protein (GFP)

Transgenic Research

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Supplementary Table 1 Ethogram for assessing sow behaviour (SB) and offspring behaviour during Open field tests (OB)

	Parameter	Description
SB + OB	Standing	motionless, all 4 feet touching floor, no other activity
	Locomotion	walking or running
	Exploring or manipulating	Nosing, sniffing, touching, licking, chewing, sucking or rooting
	Other	behaviour either not visible, not discernible without doubt or not described above
SB	Lying side	lying on the floor in lateral position with stretched limbs without other activity
	Lying belly	lying on the floor in ventral position without other activity
	Sitting	Sitting on the tail with the forelegs stretched straight under the body,
	Suckling	at least 2 piglets of the litter are are active at the udder, i.e. massaging and/or suckling
	Feeding	head dipped in the feeder or visibly ingesting/chewing food
	Drinking	snout dipped in the bowl drinker

Supplementary Table 2 Sow behaviour around 14 days post partum

	Genotype				
	GFP sows (n = 10)		Wt sows (n = 8)		P value
Behaviour	Mean (%)	SD	Mean (%)	SD	Mann-Whitney U
Standing	3.0	2.5	2.1	1.3	0.499
Locomotion	1.4	0.9	0.9	0.9	0.209
Lying side	28.8	13.1	33.4	13.3	0.571
Lying belly	19.7	8.9	19.8	7.6	0.648
Lying total	48.5	15.5	53.2	9.8	0.460
Exploring or manipulating substrate	7.5	6.2	8.4	7.3	0.845
Exploring or manipulating the pen floor or fixtures	3.8	3.1	4.6	3.6	0.613
Exploring total	11.3	8.4	13.0	8.3	0.527
Sitting	3.4	3.3	3.2	2.0	0.914
Suckling	23.3	12.9	21.0	8.5	0.713
Feeding/explore feeder	2.4	1.8	1.1	1.1	0.084
Drinking	2.0	2.0	1.2	0.9	0.623
Other	4.1	10.1	3.7	4.2	0.332

Data is represented as percentage of sample points where the respective behaviour was performed during the total observation period (360 minutes, 120 sample points).

Supplementary Table 3 Mean weight in female and male offspring and numbers of animals analysed at respective ages

	GFP animals			Wt animals		
Age (days)	Mean	SD	n	Mean	SD	n
Females						
1	1.4	0.0	37	1.5	0.0	27
7	2.6	0.1	37	2.8	0.1	27
14	4.2	0.1	37	4.8	0.1	27
21	6.0	0.2	37	6.6	0.2	27
28	8.1	0.2	37	8.7	0.2	27
35	9.8	1.1	16	10.6	1.3	14
84	43.0	3.4	8	43.1	1.8	7
119	77.6	6.2	8	80.5	5.6	7
Males						
1	1.4	0.0	49	1.5	0.0	44
7	2.5	0.1	49	2.6	0.1	44
14	4.3	0.1	49	4.4	0.1	44
21	6.0	0.2	49	6.3	0.2	44
28	8.3	0.2	49	8.3	0.2	44
35	10.2	1.1	24	10.1	1.5	24
84	41.4	2.1	12	44.1	4.7	12
119	71.6	5.6	12	75.3	8.8	11

Supplementary Table 4 Teat pair and udder region preference and fidelity in GFP and wt animals assessed during 2 sucklings in piglets at 14 days of age

	GFP animals	Wt animals	P value
Median teat pair used in suckling 1	3	3	0.970*
Median teat pair used in suckling 2	3	3	0.909*
Teat pair fidelity in percent	79	86	0.303**
Udder region fidelity in percent	92	92	>0.999**
Mean percentage of animals suckling anterior udder region	38	40	
Mean percentage of animals suckling middle udder region	51	46	
Mean percentage of animals suckling posterior udder region	11	15	

* Mann Whitney U test; ** Chi square

Supplementary Table 5 Distribution of piglets in lesion count categories for the respective body region at weaning and one week post weaning

	Count category	% GFP animals	% wt animals	P value Chi square
Head/Face at weaning	1	81	81	>0.999
	2	15	15	
	3	4	4	
Ear at weaning	1	35	32	0.473
	2	56	53	
	3	8	15	
Shoulder at weaning	1	46	29	0.083
	2	32	35	
	3	22	35	
Carpal joints at weaning	1	100	98	0.434
	2	0	2	
	3	0	0	
Flank at weaning	1	82	78	0.565
	2	15	16	
	3	2	6	
Tail at weaning	1	100	99	0.447
	2	0	1	
	3	0	0	
Head/Face one week post weaning	1	70	87	0.215
	2	13	5	
	3	18	8	
Ear one week post weaning	1	8	5	0.662
	2	31	41	
	3	62	54	
Shoulder one week post weaning	1	10	10	0.929
	2	13	18	
	3	77	72	
Front legs one week post weaning	1	100	97	0.506
	2	0	3	
	3	0	0	
Flank one week post weaning	1	43	62	0.151
	2	40	21	
	3	18	18	
Tail one week post weaning	1	100	95	0.234
	2	0	5	
	3	0	0	

Supplementary Table 6 Distribution of piglets in lesion severity categories

	Severity category	% GFP animals	% wt animals	P value Chi square
Tail	1	90	87	0.875
	2	8	8	
	3	3	5	
Ear	1	5	3	>0.999
	2	8	5	
	3	88	92	
Body	1	3	3	>0.999
	2	5	3	
	3	92	95	

Severity categories: 1 (without any lesions), 2 (only superficial scratches) and 3 (deep lesions with blood/crust) for the respective body regions.