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SITE-DIRECTED MUTAGENESIS AND GENETIC MARKERS IN SELECTION OF PIGS

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Summary

The idea behind marker assisted selection is that there are genes with significant effects that may be targeted specifically in selection. Some traits are controled by single genes. Those genes may be engineered to produce proteins that are tailored for a specific application. Site-directed mutagenesis (SDM) is used to generate mutations that can produce rationally designed protein that has improved special properties. Gene targeting using SDM has been combined with somatic cell nuclear transfer and germ cell transplantation to generate gene-targeted animal models. Targeted mutagenesis can be used to incorporated point mutation, insertion or deletion into coding ragion of a chosen gene in the epitope region. Also, promoter activity can be regulated by SDM to influence expression level of the targeting gene. SDM with gene targeting based on homologous recombination is the ultimate mutation breeding technology because it enables useful information acquired from structural-based protein engineering, to be applied directly to molecular breeding of pigs.

Key words: molecular breeding, selection, site-directed mutagenesis.

Introduction

The concept of engineering is closely tied to rational design, a process in which scientific principles are used to design a thing and then construct it. DNA technology has made it possible. Natural evolution tends to produce proteins and other molecules that are optimized for some function in their cellular environment. The development of *in vitro* evolution in a sense brings genetic engineering technology full circle. Random recombination of PCR products during co-amplification can be used to perform „sexual PCR“, in which „random“ crossovers at points of homology between co-amplified PCR products are used as an *in vitro* approximation to sexual recombination (Stupar et al., 2012a)..

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Using DNA markers and genetic engeneering in breeding livestock is increasingly spreading (Stupar et al 2012b; Vidović et al. 2012). DNA engineering methods provide the means to use selections analogous to livestock breeding at the molecular level, as a form of molecular husbandry in which variants are generated through mutation and recombination and those that best serve the desired function, such as affinity to a target, are selected and propageted. This is essentially genetic breeding of molecules, and is analogous to old-fashioned animal breeding (Vidović and Stupar, 2010), as well as in plant breeding (Stupar 1988, Stupar and Borojević 1990.).

It is important to emphasize that entire organisms can be viewed as living machines that can be engineered through the introduction of transgenes or engineered DNA molecules. Whether present on an extrachromosomal vector or integrated into a cellular chromosome, transgenes can be used to introduce new heritable characteristics into an organism while circumventing normal biological constraints. Gene sequencing, PCR amplification of the targeting gene and different technics used in Molecular Biology, like fingerprint analysis and Southern blotting can be very useful in determing what part of sequence to be changed to obtain the required quality.

Site directed mutagenesis

SDM is a molecular biology technique in which mutations is created at a deffined site in a DNA. Mutations can be done by insertions or deletions of a specific site on DNA. These minor change may have some importance if a single amino acid is aleted in a protein order to improve its properties. For such modifications point-mutations, changing of single nucleotide, is done on specific portion of the gene.

The basic SDM procedure requires the synthesis of a short DNA primer which is complementary to the template DNA around the site where the mutation is to be introduced. This synthetic primer is the gene. The gene thus copied contains the mutated site, and then introduced into

host cell as a vector and cloned. This techniques has potential for protein engineering, allowing beter protein function.

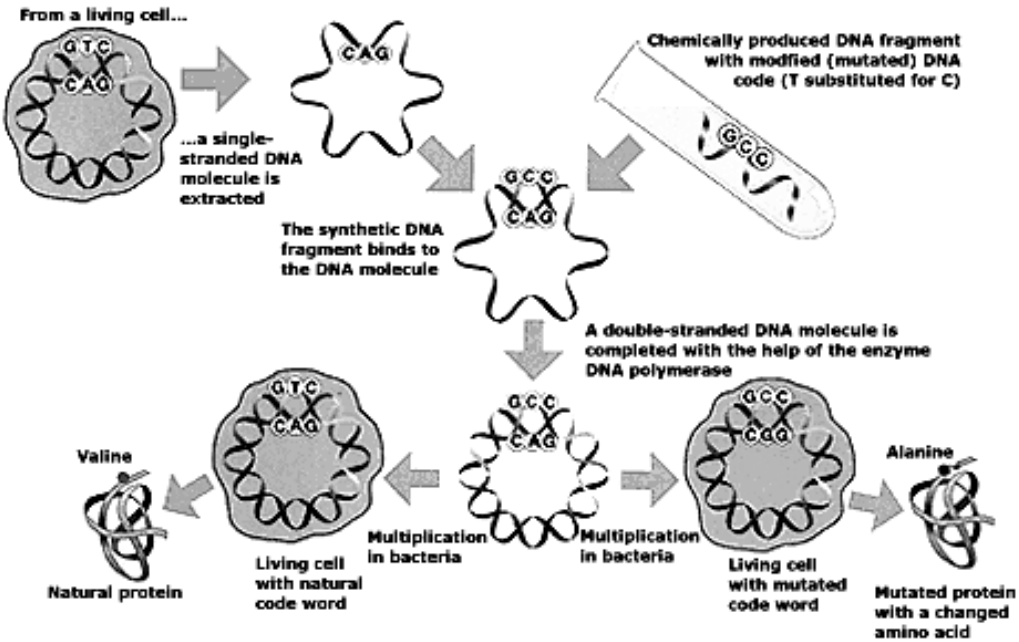
Fig. 1. shows the classical approach to the SDM invented by Michael Smith. (Hutchison et al. 1978; Kresge et al. 2006.).

The original method using single-primer extension was inefficient due to lower yield of mutants. The resulting mixture may contain both the original unmutated template as well as the mutant strand. The mutants may also be counter-selected due to presence of mismatch repair system which favors the methylated template DNA. Many approaches have since now been developed to improve the efficiency of mutagenesis. A large number of SDM are available, but since the 2000s newer techniques allow for simpler and easier way of introducing site-directed mutation into genes.

SDM, generally is used to generate mutations that may produce rationally designed protein that was improved or special properties. There are list of some of SDM techniques: Kunkel method, polymerase chain reaction (PCR) site-directed mutagenesis, cassete mutagenesis, whole plasmide mutagenesis, in vivo- SDM. SDM using in gene targeting has been combined with somatic cell nuclear transfer and germ cell transplantation to generate gene-targeted animals. The particular SDM has been achived by using recombinant adeno-associated virus, zinc-finger nuclease system, integrase lentiviral vectors et cet.

Witeen those techniques some of them are very simple and ingeniouses like: an efficinent one-step site directed deletion, insertion and miltiple site plasmid mutagenesis (Liu et al., 2008) and simple and efficient SDM using two single-primer reactions in parallel to generate mutants for protein structure-function studies (Edelheit et all., 2009).

Figure1. – USING SDM THE INFORMATION IN THE GENETIC MATERIAL CAN BE CHANGED. A SYNTHETIC DNA FRAGMENT IS USED AS A TOOL FOR CHANGING ONE PARTICULAR CODE WORD IN DNA MOLECULE. THE REPROGRAMED DNA MOLECULE CAN DIRECT THE SYNTHESIS OF A PROTEIN WITH AN EXCHANGED AMINO ACID.
Slika 1. – UPOTREBOM SDM MOGU SE PROMJENITI INFORMACIJE U GENETSKOM MATERIJALU. SINTETIČKI DNA FRAGMENTI KORISTE SE KAO SREDSTVO ZA PROMJENU GENETSKOG KODA U DNA MOLEKULI. REPROGRAMIRANA DNA MOLEKULA MOŽE DIREKTNO SINTETIZIRATI PROTEIN SA IZMJENJENOM AMINOKISELINOM.



Examples of sdm in pig's breeding

There are finite number of genes (app. 30.000 for pigs) with the „halotane“ gene being the first example of a so-called major gene in pigs (Steen et al., 2005).The increasing knowledge of the genome, from gene sequence or from expression studies, makes it possible to work on large numbers of candidate genes. This is based on the efficinet identification of polymorphisms within populatio for those genes and the

study of association between these polymorphisms (eg. SNP) and traits of interest. For example, genes within the leptin pathway represent candidate gene for feed intake, growth, fatnes. A DNA SNP was identified in the MC4R gene of pigs and found to participated in variation in production traits in several breeding lines. The polymorphism resulted in a change in an aa in a highly conserved region of the protein, suggesting that change was causative. Original mutation may be causative because different MC4R alleles differ in their respons to ligand binding using in vitro gene expression system. By analizing pig chromosome 7 (Rothschild et al., 1995). The association of the five chromosome 7 genetic markers with birth weight, weaning weight, average daily gain, back fat, loin eye size, coulor, marbling and firmness. It was shown that genetic markers S0064, TNFα, S0102, S006, S0101 on chromosome 7 were spanned approximatelz 90 cM. It was shown that TNFα were associated with difference in level of back fat, others are associated with average daily gain and loin eye area. SDM via gene targeting based on homologous recombination is the ultimate mutation breeding technology because it enables usefull information acquired from structural- and computional- based protein engineering to be applied directly to molecular breeding.

Porcine leptin affects growth hormone (GH), insulin-like growth factor-1 (IGF-1), insulin, thyroxine (T4) secretion and feed intake. Leptin receptors are expressed in the ventromedial and arcuate regions of the hypothalamus, pituitary, adipose tissue, ovary. The two primary regulators of GH secretion, GH-releasing factor (GRF) and somatostatin (SS) are produced inthe arcuate and ventromedial hypothalamus in the pig brain. These same areas involved in food intake regulation. Thus, leptin may regulate feed intake and growth in swine. Porcine leptin cDNA sequence representing the secreted porcine protein from aa 22 to 167, as a recombinant protein reduces feed intake and stimulates GH secretion in pigs (Barb et al., 1998). The discovery of the ob gene and anti-obesity effects of leptin seems to be a breackthrough in understending the role adipose tissue plays in regulating food intake, body weight, and endocrine function in swine. Studing the polymorphism

in the GH gene and its association with daily gain, meet and fat percentage, and with the regression of basal-plasma-GH concentration on age and weight it was detected polymorphism as a double-strand conformation and was assumed to be caused by an adenine being swapped with a timine in the TATA box in the GH promotor, as a quantitative trait loci for growth rate (Nielsen et al., 1995). By using SDM either in the GH or porcine leptin gene it is possible to analyse and control many pigs characteristics. Truncated form of porcine GH, esspecially C-terminal portion of the recombinant PGH, including particular the last eight AA, is of major importance in the binding of PGH to the pig liver membrane GH receptor (Puri et al., 1993).

Significant proportion of an truncated variant of recombinant pig growth hormone (rPGH) were observed on SDS-polyacrilamide gel electrophoresis relative to pig pituitary derived GH. Detailed characterisation on C-N-terminal aa sequence confirmed lacking eight C-terminal aa. This truncated form is unable to bind to the pig liver-membrane GH receptor. Consequently, this results also suggest that the C-terminal portion of rPGH, including in particular the last eight aa, is of major importance in the binding of rPGH to the pig liver membrane GH receptor (Puri et al., 1993).

A significant obstacle to cloning animals from gene-targeted primary fibroblasts is the limited life span and rapid senescence of primary fibroblasts following selection. However, long-term culturing following selection can lead to chromosome aberration, and consequently to heterogenity in the populations derived from a single cell. One of the way to solve this problem, is to rejuvenate fibroblast. Those fibroblast when can use for Southern blot confirmation, before second round of SCNT cloning to produce live animals. This two-step recloning method has also been shown to be much more efficient than direct cloning of gene-targeted cells in the generation of α 1,3-galactosyl-transferase gene knockout pigs by SCNT (Fujimura et al., 2008).

With current technology, uterine capacity (UC) represents the major limit to litter size in swine. Foetal erythropoesis may influence UC. The erythropoietin receptor (EPOR) controls the terminal differentation of

foetal red blood cells. Thus, the EPOR gene is a good candidate for association with UC. Varying the DNA sequences at promotor or epitope level of the protein is a best way to analyse the function and participation of a gene of interest into corresponding biotechnology pathway. A single nucleotide polymorphism that creates an extra GATA-1 Site (T allele) in intron 7 of the swine EPOR gene was discovered and a genotyping assay for this SNP was developed to test whether DNA sequence variation in the EPOR gene is associatet with UC in swine (Vallet, 2005.a). Positive correlation have been found with polymorphism in a quantitative trait loci and UC and litter size.

UC, or number of fetuses that can be maintained by the uterus until the end of gestation, represents the major limit to litter size in swine. Previous comparison between the cDNA and gene sequences for secreted folate binding protein (sFBP) indicate a 12-bp insertion/delition polymorphism in exon 1 and SNP that altered Ser-Arg the protein amino acid sequence. This polymorphism could be exploited to increase litter size in swine (Vallet, 2005.b).

In the table 1. is shown number of genetic markers per chromosome in porks that can be used in SDM.

TABLE 1. – NUMBER OF GENETIC MARKERS PER CHROMOSOME IN PIGS.
TABELA 1. – BROJ GENETSKIH MARKERA PO KROMOSOMU KOD SVINJA

Chromosome Kromosomi	No. markers Broj markera	Chromosome Kromosomi	No. markers Broj markera
1	94	11	40
2	75	12	39
3	90	13	88
4	84	14	95
5	57	15	79
6	106	16	41
7	98	17	22
8	108	18	20
9	62	X	61
10	52	Y	38

In the table 2. there are examples of the primer sequence and restriction enzymes (RE) that can be used in PCR analyses of pig genetic markers on reproductive traits in pigs. These primers can be used in the preparation of modified gene before insertion into the genome.

TABLE 2. – EXAMPLES OF THE DNA MARKERS AND THEIR PCR PRIMERS IN PORK
TABELA 2. – PRIMJER DNA MARKERA I NJIHOVIH PCR PRIMERA U SVINJA

Genes Gen	Primers sequence Sekvenca prajmera	RE
ESR	F: 5'-CCT GTT TTT ACA GTG ACT TTT ACA GAG-3' R: 5'-CAC TTC GAG GGT CAG TCC AAT TAG-3'	*Pvu*II
ADIPO (AJ849536)	F: 5'-TCA GGA TGC TGT TGT TGG GA-3' R: 5'-CCC TGT GAA TAG GCC TTT GG-3'	*Bsa*HI
*PEG*1	F: 5'-ATT GGC ACA GGT GAA GGG CTT TTT C-3' R: 5'-AGG CTT CAC TCG ATT AGG TCT GG-3'	*Taq*I
LEP (U66254)	F: 5'-GAG CCA ACA TCT CTC TCG CTG -3' R: 5'-GAC TCC TGG AAG CTC AGG TTT CTT C-3'	*Hin*fI

Gene transfer through addition experiments has not yet been successful in livestock improvement, perhaps mainly because expression of the transgenes such as GH could not be controled. With opportunity for specific gene modification, rather than gene insertion by transgenic technology, gene transfer is likely to use the same processus as SDM.

Proteins have been successfully improved for many years by mutation and selection, but because of long and slow nature of this process, it has not been possible to rapidly develop desired product. Molecular modeling of the protein can be powerful, but cannot be used for proteins that have not yet been crystalized, for determining protein-environment interaction, or predicting the effect of mutations beyond protein structure.

Pigs provide a valuable compatitive model to analyze implantation/placentation-associated gene and protein regulation. There is still no data-base (Chae, 2011) bearing up-to-date candidate genes and proteins for reproductive traits of pig, based on genetic similarity between human and pig and the intensive studies on human reproductive mechanism, porcine model was very valuable applied in research field about reproduction and transplantation of organs. Proteomic studies highlight the potential of protein expression profiles for developing

molecular diagnoses and for identifying molecular targets for potential breeding and therapeutic purposes.

Targeted SDM can be used to incorporate mutation, deletion or insertion into the coding region of a chosen gene-either to disrupt its expression or to produce a mutant protein that is associated with a particular human disease phenotype. Gene targeting using adeno-associated virus (AAV) and designer zinc-finger nucleases has been combining with somatic cell nuclear transfer and germ cell transplantation to generate animal models. Gene targeting can also be used to disrupt or alter cis-regulatory elements that control gene expression, or to study gene regulation through the targeted insertion of reporter genes. Transgenic mouse models have failed to replicate the human phenotype due to species-specific differences in cell biology. Pigs are consider good candidates for modeling cistic fibrosis because of similarity of their lung cell biology to that of humans.

The current success in producing gene knockout mice from genetically modified stem cells (Kanatsu et al., 2006) and from multipotent germ-line spermatogonial stem cell and from multipotent germ-line stem cells (Takehasi et al., 2007) may also become applicable to other species in the near future. A better understanding of the basic biology of male germ cells and the processes that control spermatogenesis may also provide critical clues for overcoming the remaining technical challenges to applying this technology.

In the past several years, a new technology, called DNA microarray, has attracted tremendous interests among biologists. This technology promises to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousends of genes simultaneously. There are two major forms for the DNA microarray technology: 1) Identific ation of sequence (gene/gene mutation); and 2) Determination of expression level (abundance) of genes. Microarray based gene expression profiling can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing expression in mutated to that unmutated cells or tissues. DNA microarray in combination with proteomic expression system may be powerfull methodology for application of SDM in gene targeting. (Marx, 2000).

Conclusion

Site-directed mutagenesis is an *in vitro m*ethod for creating specific mutation in known sequence, and is typically performed using PCR-based methods. Primers designed with mutations can introduce small sequence changes, and primer extension or inverse PCR can be used to achive longer mutant region. Gene sequence and its function has to be known to help determining genetic markers used in selection of pigs.

Progress in gene targeting has allowed for genetic modification of new animal genotypes (breeds), which has greatly facilitated the study of processes. Targeted mutagenesis can be used to incorporated mutation or deletion into the codin region of a chosen gene – either to disrupt its expression or to produce a mutant protein that is associated with a particular phenotype. Gene targeting can also be used to disrupt or alter *cis*-regulatory elements that control the expression of a given gene, or to study gene regulation through the targeted insertion or reporter genes. Progress in SDM associated techniques, as a gene targeting using AAV or zinc-finger nuclease, has greately facilitated the study in this direction. Aside the targeting economic characteristics, regarding progress in SDM in animal models, genetic mous models have failed to replicated human phenotype, pigs are shown new opportunities for directed genetic engineering as a model of human disease.

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CILJANA MUTAGENEZA I GENETSKI MARKERI U SELEKCIJI SVINJA

Sažetak

Ideja koja stoji iza selekcije potpomognute genetskim markerima je da postoje geni sa signifikantnim učinkom koji se mogu upotrijebiti u ciljanom izboru svojstava. Neke osobine su kontrolirane jednim genom, koji su odgovorni za sintezu specifičnog proteina. Ciljana mutageneza (SDM) se upotrebljava da bi se proizvele mutacije koje daju racionalno dizajniran protein. SDM se kombinira sa transferom jezgre somatskih stanice kao i transplantacijom germ stanica pri dobivanju životinjskog modela u kojima su promijenjeni odgovarajući geni. Ova mutageneza se koristi za ubacivanje točkastih mutacija, insercija ili delecija u funkcionalnom dijelu, epitopu, datog gena. Također, aktivnost promotora može se regulirati putem SDM i tako utjecati na razinu ekspresije gena od interesa. Kombinacija SDM sa homolognom rekombinacijom gena od interesa je kao oplemenjivačka tehnologija budući da omogućuje uporabu korisnih informacija zasnovanih na proteinima koji se direktno koriste u molekulskom oplemenjivanju svinja.

Ključne riječi: molekulsko oplemenjivanje, selekcija, site-directed mutageneza, genetski markeri kod svinja.

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