Ontogeny of LH receptor gene expression in the pig reproductive tract

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In view of the recently documented expression of the LH receptor gene in several non-gonadal reproductive tissues, the aim of this study was to analyse further the ontogeny of expression of this gene in the pig reproductive tract in fetal and neonatal life. RT-PCR was used to investigate the expression of the extracellular and transmembrane receptor domains, and to identify the time of onset of transcription of the full-length LH receptor mRNA and its shorter splice variants. Expression of the LH receptor gene was first detected around day 30 of fetal life in both ovary and testis, coinciding with morphological differentiation. The pattern of expression of LH receptor splice variants did not change during postnatal gonadal maturation. Expression of the LH receptor gene in the pig non-gonadal reproductive tract started during fetal life and continued during sexual maturation. A novel pig LH receptor spliced variant, lacking exon 10, was detected for the first time. The transmembrane receptor domain was expressed in fetal tissues, but not in neonatal control tissues. On the basis of the transmembrane domain of the LH receptor mRNA, it is concluded that the ovary and extragonadal tissues of the pig reproductive tract, like the pig testis, synthesize functional LH receptors during fetal life. The presence of LH receptor mRNA in extragonadal reproductive tissues indicates that LH is involved in the control and regulation of reproductive tract maturation.

Introduction

Several mammalian non-gonadal reproductive tissues express the LH receptor gene. In pigs, such tissues include the oviduct (Ziecik *et al.*, 1992), endometrium, myometrium (Ziecik *et al.*, 1986) and the broad ligament (Ziecik *et al.*, 1995). In mice (Adams and Brumlow, 1989), rat, monkey (T Zhang *et al.*, 1997) and human male accessory sex organs, LH receptor is present in the epididymis, as well as in the rat prostate (Reiter *et al.*, 1995; Rao, 1996). The significance of extragonadal LH receptor expression remains elusive. However, the LH receptor found in human non-gonadal tissues (Rao 1996) and in pig myometrium appears to be functional. In pig myometrial cells, hCG activates two different second messenger signalling systems, that is, adenylate cyclase and phospholipase C (Kisielewska *et al.*, 1996). On the basis of tissue localization and functional properties, the LH receptor expression in non-gonadal reproductive tissues appears to be closely related to preparation of the reproductive tract for fertilization, followed by growth, development and implantation of the blastocyst. It also appears to be related to the maintenance of uterine quiescence and the increase of uterine blood flow in pregnancy (for review see Rao, 1996).

The gonadal LH receptor is localized in theca, granulosa, luteal and interstitial cells in the ovary, as well as in Leydig

cells in the testis (Catt and Dufau, 1976), and plays a key role in the regulation of gonadal steroidogenesis. Leydig cells express the LH receptor constitutively in the absence of hormonal stimulation (Huhtaniemi 1994), whereas ovarian LH receptor expression is enhanced by the appropriate hormonal stimulation during the ovarian cycle. In pigs, testicular LH receptor can be found during early fetal development (Hennen *et al.*, 1982), and its appearance coincides with the onset of steroidogenesis. The fetal testes of many mammalian species commence active testosterone production as soon as the Leydig cells are morphologically differentiated (Huhtaniemi, 1994). In contrast, the mammalian fetal ovary, including that of the pig (Elsaesser and Parvizi, 1979), is endocrinologically quiescent during the perinatal period.

The onset of LH receptor expression and function in developing rat gonads is well established (Sokka *et al.*, 1992; Zhang *et al.*, 1994). In both sexes, the LH receptor expression starts with the appearance of mRNA encoding extracellular receptor domains around the time of morphological differentiation of the gonads, that is, day 15 after mating. Subsequently, expression of full-length LH receptor mRNA starts in conjunction with the onset of ovarian endocrine function on day 7 post partum (Sokka and Huhtaniemi, 1990), and Leydig cell steroidogenesis around day 15.5 after mating (Zhang *et al.*, 1994). The neonatal mouse ovary displays a similar expression pattern. The extracellular domain of the LH receptor is expressed before mRNA encoding the full-length

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Received 3 July 1998.

Fig. 1. Pig LH receptor cDNA isoforms (pLHR A–D) aligned from the first methionine codon. LH receptor exons are shown as numbered boxes. The splice site junction between exons 10 and 11, in which the divergence appears, is shown as a dotted line. The LH receptor specific primers for RT–PCR were designed according to the organization of exons and deletions in the isoforms. The expected RT–PCR products are shown as lines between the pairs of primers (oligo 4 and 5 and oligo R1 and R2) and the predicted length is given above the lines.

receptor appears, and it is correlated with postnatal ovarian development (O’Shaughnessy *et al.*, 1997).

The aim of the present study was to characterize expression of the different LH receptor gene domains at early stages of fetal development in pig gonads, non-gonadal reproductive tissues and non-reproductive control tissues, and to compare the appearance of the extracellular and transmembrane receptor domains.

Materials and Methods

Tissue collection

Fetal reproductive tracts and control tissues were collected from male and female piglets of the Duroc breed (duration of pregnancy = 114 days) on days 30, 40, 48, 70, 100 and 103 after mating (day of oestrus = day 0), immediately after the sows were slaughtered, and on days 1, 40, 50, 100 and 180 of postnatal life. Reproductive tracts from postnatal animals were collected, stored and analysed individually. Pairs of fetal gonads were analysed separately. The tissues were frozen immediately in liquid nitrogen and stored at –70°C until RNA isolation. The intestine, kidney and liver from fetal and adult animals, and fetal heart and adult muscle were used as negative control tissues, and adult corpora lutea and testes as positive controls.

RNA isolation and RT–PCR

Briefly, total RNA was isolated according to Chomczynski and Sacchi (1987), and Puissant and Houdebine (1990). The

purity of RNA was determined spectrophotometrically by A₂₆₀:A₂₈₀ ratio and by agarose–formaldehyde gel electrophoresis (Sambrook *et al.*, 1989). Variants of the pig LH receptor cDNA (Loosefelt *et al.*, 1989) from Gene Bank (pLHRA, pLHRB, pLHRC, pLHRD) were aligned and oligonucleotides for RT–PCR were designed using the DNASTar primer selection program (DNASTAR Inc., Madison, WI). Primer positions were counted from the first methionine codon. Two sense primers, oligo R1 (5’-CTCAGAGTGATTCCC-3’, nucleotides 245–256 in exon 3) and oligo 4 (5’-GACGCTAATTGCCACATCATCCTA-3’, nucleotides 738–761 in exon 9), and two antisense primers, oligo R2 (5’-TACCCCAGCCACTGA-3’, nucleotides 823–837 in exon 9) and oligo 5 (5’-ATTATGCTTGGAGGGTGGCT-3’, nucleotides 1456–1475 in exon 11), were chosen (Fig. 1). The specificity of the primers was confirmed by the BLAST program (Altschul *et al.*, 1990).

RT–PCR was performed according to a coupled one-step procedure (Aatsinki *et al.*, 1994). Total RNA (2 µg per 100 µl reaction mixture) was reverse transcribed at 42°C for 1 h, denatured at 97°C for 3 min, and amplified for 30 cycles, including denaturation at 96°C for 30 s, primer annealing at 55°C for oligo R1 and R2 and 56°C for oligo 4 and 5 for 30 s, and extension at 72°C, with the elongation time option (10 s per cycle). The enzymes used were avian myeloblastosis virus reverse transcriptase, RNasin (Promega, Madison, MI) and Taq DNA polymerase (DynaZyme, Finnzymes, Finland). The PCR products (20 µl) were separated by electrophoresis on 1.5% (w/v) agarose gel and photographed under UV light. Alignment of the pig LH receptor cDNA sequences allowed the number and size of the expected RT–PCR products to be predicated (Fig. 1).

Determination of gonadal sex at early stages of development

DNA contamination in 2 µg RNA samples was used to determine the sex of the fetal gonads on days 30 and 40. The presence of male-specific repeated DNA sequence (accession no X12696 by McGraw *et al.*, 1988) was investigated according to Goxe *et al.* (1993) using two PCR primers, sense 5'-TACTGGGAGTGTGGTGCCTGGGGC-3' and antisense 5'-CCCTGGGAGCTCTGGCTTCA-3'. Briefly, 100 pmol each primer µl⁻¹, 2 U DynaZyme polymerase, 200 µmol each deoxyribonucleotide l⁻¹, and 2 µg total RNA in a final volume of 100 µl were denatured at 97°C for 30 s and subjected to 30 cycles of PCR with denaturation at 95°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 1 min; the last elongation was carried out for 10 min. PCR products were separated by electrophoresis on a 1% (w/v) agarose gel and photographed under UV light.

Southern hybridization

RT-PCR products (20 µl) were separated by electrophoresis on 1.5% (w/v) agarose gels and transferred to nylon filters (Hybond N, Amersham) using standard procedures. Hybridization was performed according to Sambrook *et al.* (1989) with a pig cDNA probe labelled with [α -³²P]deoxycytidine 5'-triphosphate (dCTP) using the random prime method (Multiprimer DNA Labelling System, Amersham). Products corresponding to the extracellular LH receptor domain were hybridized with fragment 260–900, and products of oligos 4 and 5 with fragment 900–2081 of pig LH receptor cDNA (pLHRA) at 65°C for 12 h. After hybridization, the blots were washed twice with 1 × SSC (150 mmol NaCl l⁻¹, 15 mmol sodium citrate l⁻¹, pH 7.0) containing 0.1% (w/v) SDS for 15 min at room temperature, and twice with 0.2 × SSC containing 0.1% (w/v) SDS for 15 min at 65°C. The washed sheets were exposed to Fuji X-ray film at –80°C for 12–20 h or 10 days (negative controls).

Sequencing

RT-PCR products representing the fragment spanning exons 9–11 were subcloned into the T-vector (Promega, Madison, MI) according to the manufacturer's instructions and sequenced from both strands using a Perkin Elmer automatic sequencing machine (Perkin Elmer Inc., Foster City, CA). The results were analysed with the DNASTar Program (DNASTAR Inc., Madison, WI) to confirm the identity of the sequence.

Northern hybridization

Northern hybridization was performed using 20 µg total RNA isolated from neonatal gonadal and non-gonadal tissues, and negative fetal tissues. Samples were separated by electrophoresis on a 1.2% (w/v) denaturing agarose gel and transferred onto nylon membrane (Hybond N). Prehybridization and hybridization were carried out according to the manufacturer's instruction. Briefly,

membranes were prehybridized for 4 h at 64°C in a solution of 3 × SSC, 50% (v/v) deionized formamide, 5 × Denhardt's solution, 0.1% heat-denatured calf thymus DNA l⁻¹, 1% (w/v) SDS and 100 mg yeast tRNA l⁻¹. Hybridization was performed at 66°C overnight after addition of the [³²P]-labelled cRNA probe to the prehybridization solution. After hybridization, membranes were washed twice in 2 × SSC containing 0.1% (w/v) SDS at room temperature, once in 1 × SSC and 0.1% (w/v) SDS for 10 min at 65°C and once in 0.1 × SSC and 0.1% (w/v) SDS for 10 min at 65°C. The filters were exposed to X-ray film at –70°C for 5–14 days.

[³²P]-labelled cRNA probe was synthesized using the Riboprobe system (Promega, Madison, MI). A full-length pig LH receptor cDNA (nucleotides –11 to 2089 pLHRA) in Bluescript vector was used as a template (Le Kremlin, Bicetre).

Results

Sex determination of gonads

The slight genomic DNA contamination present in 2 µg total gonadal RNA was sufficient for sex determination by PCR of fetal gonads in early stages of development. Male samples were characterized by a 500 bp PCR product.

Expression of the LH receptor gene

Expression of the extracellular domain of the LH receptor was investigated using oligo R1 and R2 primers, corresponding to exons 3 and 9, respectively. Southern hybridization of the PCR products revealed that both sexes displayed transcripts for the extracellular region as early as day 30 of fetal life (one of two female samples and three of five male samples). Samples of both sexes produced the expected RT-PCR amplicon of 600 bp, as well as an additional product of 440 bp (Fig. 2). A shorter cDNA product from this region has been found in previous studies (Ziecik *et al.*, 1992; Derecka *et al.*, 1995; Derecka and Ziecik, 1997).

The same gonadal samples were then analysed for transcripts of exons 9–11 (extracellular and transmembrane), and two of two female, and three of five male gonads expressed this region on day 30 of fetal life. All gonads on day 40 expressed exons 9–11 (Fig. 2). Embryonic gonads displayed the same pattern of RT-PCR products as corpus luteum (Fig. 3). A computer prediction indicated two products for the oligonucleotide pair 4 and 5 (Fig. 1), one of 740 bp based on isoform A and the other of 470 bp corresponding to sequence B from Gene Bank. RT-PCR revealed an additional product of 670 bp. The bands of 740 and 670 bp were distinguished by electrophoresis on 1.5% (w/v) agarose gels overnight. As a final confirmation of size and sequence specificity, the products of the oligonucleotide pair 4 and 5 were subcloned into the T-vector and sequenced. The product of 670 bp was found to be a novel isoform without exon 10. This product was also present in the corpus luteum, testis and extragonadal tissues (Figs 2 and 5).

Fig. 2. Expression of the LH receptor gene in pig gonads in the early stages of fetal development, as measured by RT-PCR followed by Southern hybridization. (a) The extracellular (Ex) domain amplified between exons 3 and 9 with oligo R1 and R2 primers. (b) The transmembrane (TM) domain amplified between exons 9 and 11 with oligo 4 and 5 primers. The sex of the gonads was determined by PCR according to Goxe *et al.* (1993). f, female; m, male.

Fig. 3. Expression of LH and hCG receptor gene measured by the presence of transcripts encoding the transmembrane (TM) domain in pig epididymis during fetal life. The region was amplified with oligo 4 and 5 primers, corresponding to the sequence between exons 9 and 11.

The expression of both extracellular and transmembrane regions was shown in the ovary and testis on day 70 of fetal life (Fig. 4). The uterus expressed the transmembrane domains by day 40 of fetal life (Fig. 5), and the epididymis samples were positive at both fetal ages studied, that is, 70 and 100 days (Fig. 3).

The control tissues such as the fetal liver, heart and intestine did not display extracellular domain transcripts, but did reveal the presence of RT-PCR products corresponding to exons 9–11 using oligo 4 and 5 primers (Fig. 6). mRNA from adult muscle, intestine, kidney and liver was amplified using the same primers (oligo 4 and 5) for exons 9–11 of the LH receptor, and no RT-PCR products were detectable on X-ray film after 20 h to 10 days of exposure (Fig. 6, lower panel).

During postnatal life, transcripts encoding extracellular and transmembrane domains were found by day 1 in the uterus, and the epididymis samples were positive on day 40 of fetal life, which was the earliest age studied. Uterus on day 50 and epididymis on day 180, as well as adult epididymis, were negative for the 670 bp product (Fig. 7).

Northern hybridization

Multiple species of LH receptor transcripts were found on northern blot analysis of the various reproductive tissues (data not shown). The neonatal ovary and testis showed two major bands of 4.7 and 1.4 kb. The same major band of 4.7 kb was present in the uterus and epididymis, as well as in the control tissues, that is, fetal liver, intestine and kidney. The 1.4 kb transcript was abundant in ovary and testis, less abundant in uterus and epididymis, and absent in control tissues. The minor band of 2.6 kb was characteristic of ovary, testis and uterus, as well as fetal liver, intestine and kidney. Transcripts of 4.0 kb were present in the ovary, in the developing testis from day 1 to day 100, and also in the epididymis from day 180.

Discussion

The RT-PCR results show that in the pig ovary and testis, expression of the LH receptor gene starts around day 30 of fetal life, shortly after morphological differentiation of the testis. These findings extend earlier studies on the ontogeny of LH receptor gene expression in male and female reproductive tissues (Derecka and Ziecik, 1997) and are in agreement in part with the data of Goxe *et al.* (1993) and Ziecik *et al.* (1990a). Some of the gonads from day 30 of fetal life did not show expression, probably due to variation in fetal position in the uterus and nutrition, or to individual differences in the development of fetuses. Therefore, day 30 after mating is around average for the onset of expression of the LH receptor in pig fetal gonads.

In rats, the extracellular domain is constitutively expressed in fetal gonads (Sokka *et al.*, 1992; Zhang *et al.*, 1994) and in precursor Leydig cells of the adult testis (Tena-Sempere *et al.*, 1994). Expression of the transmembrane

Fig. 4. Southern blot analysis of RT-PCR products from pig gonads on day 70 of fetal life investigated for expression of the LH receptor gene. (a) The cDNA fragment flanked by oligo R1 and R2 primers from the extracellular (Ex) domain. (b) The transmembrane (TM) domain amplified with oligo 4 and 5 primers, corresponding to the sequence between exons 9 and 11.

Fig. 5. RT-PCR products derived from total RNA isolated from pig uteri during fetal life hybridized with pig LH receptor cDNA probes. (a) The cDNA fragment flanked by primers from the extracellular (Ex) domain (oligo R1 and R2). (b) The transmembrane (TM) region amplified with oligo 4 and 5 primers, corresponding to the sequence between exons 9 and 11.

domain starts later, signalling the onset of functional LH receptor expression. The present results show that the transmembrane domain is expressed constitutively in the ovary and testis during pig development. Therefore, the developmental expression of the different LH receptor splicing variants does not appear to follow the same pattern in different mammalian species.

In pigs, maternal recognition of pregnancy occurs by oestrogens secreted by the uterine blastocyst. The highest concentration of oestradiol sulfate in maternal blood occurs

on day 26 of pregnancy (Robertson and King, 1974), which coincides with the period of morphological differentiation of the fetal testis (Pelliniemi, 1975, 1985). Interestingly, the pig placenta expresses pig steroidogenic acute regulatory protein (pStAR) and pig P450 side chain cleavage (pP450scc) (Pilon *et al.*, 1998) is involved in regulation of steroidogenesis in known steroidogenic tissues. According to Pilon *et al.* (1998), pig steroidogenic factor 1 (pSF1) expression correlates with expression of pStAR and pP450scc. This raises the question as to whether oestradiol, as the fetal signal of pregnancy, and oestradiol-regulated factors can act at the same time as the factor that triggers expression of the LH receptor gene in the fetal reproductive system.

In pigs, both the testis and the ovary expressed the extracellular and transmembrane LH receptor domains during postnatal development. Ovary and testis displayed the same RT-PCR products during sexual maturation as the corpus luteum. SF1 is involved in sex differentiation of pig gonads in both the steroid and protein hormonal concentrations. Expression of the pSF1 gene starts around the time of gonadal differentiation and is restricted to the testis during prenatal life, and then takes place in adolescent and adult testes, while only adult ovaries express pSF1 (Pilon *et al.*, 1998). This indicates that LH receptor gene expression may be mediated by different factors in the ovary and testis during fetal life and gonadal maturation.

In this study, the extragonadal tissues of the reproductive tract, for example the uterus and epididymis, were investigated for LH receptor expression during fetal and neonatal life. The results indicate that, in the uterus, the transmembrane domain is expressed from day 40 of fetal life. The epididymis expressed the transmembrane domain on days 70 and 100 of fetal life. Assuming that the presence of the transmembrane transcripts of the LH receptor gene indicates the ability to express functional LH receptor, both gonadal and extragonadal reproductive tissues of the pig reproductive tract have this ability in fetal life.

During postnatal development, LH receptor was expressed in both the uterus and the epididymis. Transcripts of the transmembrane domain (RT-PCR products) and the major transcript of 4.7 kb in total RNA (northern hybridization) were expressed permanently in extragonadal tissues. Expression of the extracellular domain was not detected in the uterus on day 50. This difference in the amount of LH receptor transcripts can be correlated with the hormonal status of the different sexes at this time in development. Females have low oestradiol concentrations in the circulation, whereas males have high concentrations of both oestradiol and testosterone (Ziecik *et al.*, 1989, 1990b). In addition, these tissues have different origins (the uterus originates from the Müllerian duct and the epididymis from the Wolffian duct) and LH receptor gene expression can be regulated in a tissue-specific manner, involving tissue- or cell-specific transcription factors or even discrete differences in the LH receptor gene promoter. This is supported by tissue-specific regulation of human LH receptor genes I and II (Tsai-Morris *et al.*, 1998). These genes have different promoters and exon 1 sequences and, most importantly, gene I is active in the testis, while gene II is active in the ovary.

The role of the functional LH receptor in pig ontogeny is

Fig. 6. Controls for the RT-PCR with pig reproductive tract tissues. Negative control tissues were heart (h), liver (l), intestine (i), kidney (k) and muscle (m). Corpus luteum (cl) was used as a positive control. Control reaction mixtures were amplified without reverse transcriptase (-RT). (a,b) Positive and negative tissues from days 30, 40 and 48 of fetal life and (c) adult tissues were tested for LH receptor gene expression. The extracellular (Ex) domain was amplified with oligo R1 and R2 primers (a), and for the transmembrane (TM) domain, oligo 4 and 5 primers from exons 9–11 were used (b,c).

unclear. In fetal rat (Majdic *et al.*, 1998) and mouse (O'Shaughnessy *et al.*, 1998) testes, the onset of steroidogenesis is an LH-independent process and Leydig cells start to be gonadotrophin dependent shortly after birth. In the early stages of development, that is, early folliculogenesis to the primary stage, the mouse neonatal ovary is gonadotrophin independent and then becomes gonadotrophin regulated (O'Shaughnessy *et al.*, 1997). The ability of the pig ovary to express the LH receptor during fetal life when the ovary is endocrinologically quiescent indicates that there are different control mechanisms for gonadotrophin receptor development in the gonads.

Lack of LH receptor function results in serious disorders in the differentiation of genitalia, the development of puberty and in fertility. Mutations causing a loss of function in the LH receptor cause pseudohermaphroditism in men (Misrahi *et al.*, 1997; Latronico *et al.*, 1998) and primary

amenorrhoea in women (Latronico *et al.*, 1998). The epididymis is essential for sperm maturation and storage, and LH controls epididymal differentiation, function and sperm maturation through its receptors and discrete regulatory factors (T Zhang *et al.*, 1997). In the female reproductive system, the LH receptor is involved in co-ordination of differentiation and maturation of the uterus.

Temporary expression of the transmembrane but not the extracellular receptor domain was also found in control tissues such as the heart, liver, kidney and intestine during fetal but not neonatal life. Such changes in gene expression during ontogeny were observed in mouse smooth muscle-specific protein SM 22 (Moessler *et al.*, 1996). Positive RT-PCR reactions for the LH receptor in control tissues have been reported in other studies, for example, in the rat kidney (Pietilä *et al.*, 1996) and the amphibian liver (Kubokawa and Ishii, 1987).

Fig. 7. Southern blot analysis of expression of the LH receptor gene in pig extragonadal reproductive tissues during postnatal life. (a) The extracellular (Ex) domain was investigated with oligo R1 and R2 primers and (b) the transmembrane (TM) domain with oligo 4 and 5 primers. Pig LH receptor cDNA was used as a probe.

The RT-PCR results of the present study revealed the presence of a novel LH receptor splice variant lacking exon 10. This transcript was not found in rats or humans, but was reported in sheep (Bacich *et al.*, 1994), and is the major LH receptor RNA species in the marmoset monkey testis (FP Zhang *et al.*, 1997). According to Remy *et al.* (1996), this exon is not important for hormone binding inhibition or stimulation of cAMP production. Exon 10 may be involved in folding of the extracellular domain of the receptor molecule and may not play any role in hormone binding. It might also be involved in specific signal transduction mechanisms of the LH receptor that are different from classical cAMP generation (Fernandez and Puett, 1996; Kudo *et al.*, 1996). This proposal is supported by the work of FP Zhang *et al.* (1997), demonstrating that the marmoset monkey testis expresses LH receptor protein that is totally devoid of the sequence encoded by exon 10. However, this receptor is able to bind hormones and to activate the second messenger system. Conflicting data were presented by Roche *et al.* (1992), which indicated that at least part of the LH receptor sequence encoded by exon 10 participates in hormone binding. In the present study, the absence of an isoform lacking exon 10 in the uterus on day 50, in the epididymis on day 180, in the adult boar and in fetal tissues, indicates that alternative splicing of the LH receptor mRNA may be under control during ontogeny.

The authors thank P. Pakarinen for helpful discussions and for advice during these experiments, and J. Klos, G. Golba and M. Blitek for technical assistance during tissue collection. They also thank A. P. F. Flint for a critical review of the manuscript. Bluescript vector was kindly donated by H. Loosfelt, Le Kremlin, Bicetre, France. This paper was partially supported by grant No. 5S31001405 from the State Committee for Scientific Research (KBN), Poland, to K. Dereckal, and by a grant from the Centre for International Mobility in Finland.

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