A tissue- and gender-specific regulation of the SARS-CoV-2 receptor ACE2 by p53 in pigs

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ABSTRACT

The current COVID-19 pandemic is caused by infections with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A sex-bias has been observed, with increased susceptibility and mortality in male compared to female patients. The gene for the SARS-CoV-2 receptor *ACE2* is located on the X chromosome.

We previously generated *TP53* mutant pigs that exhibit a sex-specific patho-phenotype due to altered regulation of numerous X chromosome genes. In this study, we explored the effect of p53 deficiency on ACE2 expression in pigs. First, we identified the p53 binding site in the *ACE2* promoter and could show its regulatory effect on *ACE2* expression by luciferase assay in porcine primary kidney fibroblast cells. Later, quantitative PCR and western blot showed tissue- and gender-specific expression changes of ACE2 and its truncated isoform in p53-deficient pigs. We believe these findings will broaden the knowledge on *ACE2* regulation and COVID-19 susceptibility.

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1. Introduction

The first outbreak of coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was reported in Wuhan, China, in December 2019 [1,2]. The most common symptoms of COVID-19 are manifested by fever, dyspnea, dry cough, myalgia, diarrhea, and pneumonia [3,4]. In severe cases, symptoms like acute respiratory distress syndrome, arrhythmia, acute cardiac injury, and acute kidney injury may occur, which likely can lead to death [3]. SARS-CoV-2 shares 79.5% sequence identity with SARS-CoV, both of which recognize angiotensin-converting enzyme-2 (ACE2) as their host receptor to cell entry [5]. The binding affinity of SARS-CoV-2 to the ACE2 receptor is 10–20 times higher than that of SARS-CoV [6]. The expression of *ACE2* varies greatly between individuals [7], but higher ACE2 concentration in plasma of men than women was reported [8]. Susceptibility of spermatogonial cells and prostate

endocrine cells to SARS-CoV-2 infection also suggests male-specific vulnerabilities [9]. Recently, Onabajo et al. found a novel truncated ACE2 isoform, which likely maintains ACE2 enzymatic function in the renin-angiotensin system [10].

Cancer patients with COVID-19 disease more often have severer symptoms though no clear association between cancer and COVID-19 infection has so far been found [11]. The p53 transcription factor is a tumour suppressor and the *TP53* is the most mutated gene in human cancer [12,13]. Loss of p53 deregulates several genes located on the X chromosome [14]. *ACE2* is located on the X chromosome in mammals and could alter the expression of p53 in pulmonary endothelial cells after lung injury [15].

In the study, we explored a tissue- and gender-specific expression of ACE2 in p53-deficient pigs.

2. Materials and methods

2.1. Animals

Two months old homozygous *flTP53^{R167H/R167H}* female and male pigs (n = 3 of each gender) and matched wild type (WT) pigs (n = 3 of each gender) were produced in our research farm. The generation of the *flTP53^{R167H}* pig line has been previously described [16]. Briefly, the *flTP53^{R167H}* pigs carry a Cre-removable transcriptional

stop signal in intron 1 and an oncogenic mutation R167H in exon 5. The intact allele lacks *TP53* expression. Tissue samples from various organs (n = 13) were collected post mortem, snap-frozen in liquid nitrogen, and stored at −80 °C. All experiments on animals conformed to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals and were approved by the Government of Upper Bavaria (55.2-1-54-2532-6-13).

2.2. Vector construction

The porcine *ACE2* promoter sequence was obtained from GenBank (NM_001123070.1). The promoter sequence from −787bp to +24bp related to the transcription start site (TSS), which included the *in silico* (JASPAR development server) predicted p53 binding site, was amplified by GoTaq DNA polymerase (Promega, Germany) with forward primer: CCTTGTGACCTTGGCTGAGT and reverse primer: AAGGAGCCAGAAAGAGCCTG. Two expression vectors were designed that carried luciferase reporter gene driven by *ACE2* promoter with (ACE2p53-con) and without *TP53* binding site (ACE2p53-ko) (Fig. 1a). The predicted *TP53* binding site (−317bp to −303bp) in the ACE2p53-ko vector was removed from the *ACE2* sequence by overlap extension PCR. The two *ACE2* promoter variants were incorporated in front of the reporter gene by the NEBuilder HiFi DNA Assembly Cloning Kit (NEB, USA). The TK promoter served as an internal control for normalising the activity of the *ACE2* promoter.

2.3. Cell culture and treatment

Porcine kidney fibroblast (PKDNF) cells were isolated from the kidney of WT pig (German Landrace). Cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA), 2 mM L-Glutamine, non-essential amino acids, and incubated at 37 °C with 5% CO_2 in a humid environment. The PKDNF cells were transfected with ACE2p53-con and ACE2p53-ko expression vectors by Lipofectamine 2000 Transfection Reagent (Thermo Fischer). 36 h post-transfection, cells were lysed with passive lysis buffer, and luciferase activity was measured using the *Firefly & Renilla* Luciferase Single Tube Assay Kit (Biotium) by FLUOstar Omega (BMG Labtech).

2.4. Quantitative PCR

Total RNA from all tissue samples was isolated using RNA Extraction Kit (Qiagen, Germany) and 500 ng of total RNA was reverse transcribed using Superscript IV (Thermo Fisher). SYBR-Green PCR Master Mix (Kapa Biosystems Pty, South Africa) was used for the quantitative PCR (qPCR). Following qPCR primers were used: *ACE2*_forward: AAGGATATTCGAGGAGGCTG; *ACE2*_reverse: CAGGAAGTCATCCATCGTCA; *GAPDH*_forward: CTCAACGAC-CACTTCGTCAA, *GAPDH*_reverse: CCCTGTTGCTGTAGCCAAAT.

2.5. Western blot

Protein was isolated by NP40 buffer with protease inhibitor and the concentration was measured by a Bradford Reagent (Sigma-Aldrich). An equal amount of proteins was loaded on 12% SDS-PAGE gel. ACE2 antibody (AF933; R&D Systems/Bio-Techne) was diluted at 1:500 and GAPDH antibody (G8795; Sigma-Aldrich) was diluted at 1:1000. To ensure accuracy, the proteins from female and male animals were loaded in the same chamber and transferred to the same membrane. The blots were exposed to the same film to keep the exposure time equal.
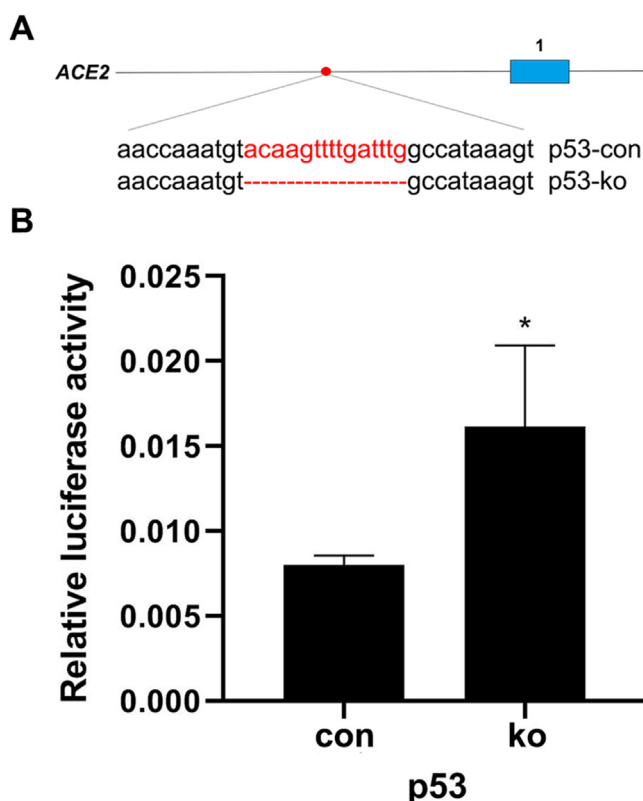

Fig. 1. Effects of p53 on the porcine *ACE2* promoter activity. (**A**) The location of the predicted with JASPAR p53 binding site in *ACE2* sequence. The porcine *ACE2* promoter sequence was obtained from GenBank (NM_001123070.1). The predicted *TP53* binding site is located between −317bp and −303bp related to the transcription start site. (**B**) The relative luciferase activities of ACE2p53-con and ACE2p53-ko vectors in porcine kidney fibroblast cells. The luciferase activities were measured 36 h post-transfection.

2.6. Statistics

Experiments in this study were conducted in three independent repeats. SPSS 22.0 was used to calculate the *p* values with the Student T-test. *$p < 0.05$, **$p < 0.01$.

3. Results

3.1. Effect of p53 on the activity of ACE2 promoter

To explore the regulation of *ACE2* by p53 transcription factor, porcine PKDNF cells were transfected with ACE2p53-con and ACE2p53-ko dual luciferase expression vectors. The relative luciferase activity was approx. twofold higher ($p < 0.05$) in PKDNF transfected with ACE2p53-ko vector, which lacks the p53 binding site, than in those transfected with ACE2p53-con (Fig. 1b). This finding indicated that the predicted p53 binding site is involved in the regulation of *ACE2* in pig kidney.

3.2. The expression profiles of ACE2

To get a broader picture for the regulation of *ACE2* by p53, we analysed the ACE2 expression in 13 different organs (colon, kidney, heart, liver, lung, spleen, small intestine, stomach, muscle, lymph node, esophagus, aorta, and brain) in female and male *flTP53*^{R167H} and WT pigs by qPCR and western blot. Since the lung is the main target organ of COVID-19 [17], the *ACE2* mRNA expression was normalised to the expression in the lung of female WT pigs. Overall,

the mRNA expression of *ACE2* was higher in female than male WT pigs, with the highest levels in the kidney and small intestine (Fig. 2a and b). However, the ACE2 protein expression in the kidney and small intestine was higher in WT males (Fig. 2c and d). This discrepancy can be explained by the posttranslational ACE2 modifications. Notably, we detected two ACE2 isoforms, the full length (~120 kD) and the truncated (~58 kD). The ACE2 full-length isoform

was expressed in most of the tissues in females, and only in kidney, liver and small intestine of male WT pigs (Fig. 2c and d).

In *flTP53^{R167H}* pigs, we observed tissue- and gender-specific ACE2 expression changes (Fig. 2a–d). In *flTP53^{R167H}* females, the expression of the full-length ACE2 isoform was higher than WT females in most tissues, particularly in small intestine and kidney (Fig. 2c, e). In *flTP53^{R167H}* males, the expression of ACE2 was higher
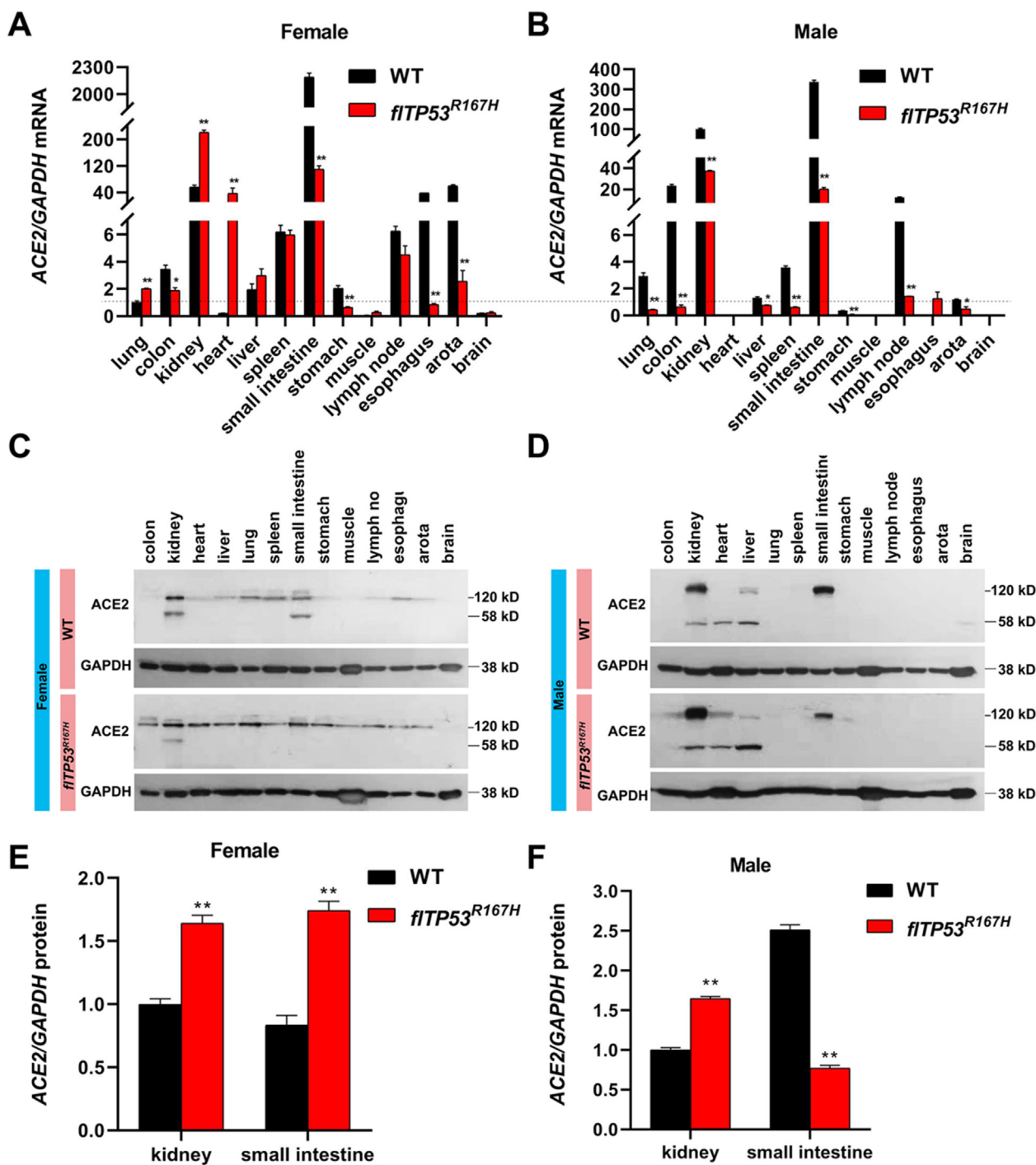

Fig. 2. A tissue- and gender-specific expression profile of ACE2 in pigs. Quantitative RT-PCR analysis of different tissues (n = 13) from female (**A**) and male (**B**) two-month-old *flTP53^{R176H}* knockout (n = 3) and wild type (n = 3) pigs. Representative western blots showing ACE2 protein expression in different tissues (n = 13) from female (**C**) and male (**D**) two-month-old *flTP53^{R176H}* knockout and wild type pigs. The full length (120 kD) and the truncated (58 kD) ACE2 isoforms were detected. Quantitative measurements of the full-length ACE2 protein expression in kidney and small intestine from female (**E**) and male (**F**) two-month-old *flTP53^{R176H}* knockout (n = 3) and wild type (n = 3) pigs. The GAPDH expression was used as the reference. *P < 0.05, **P < 0.01.

in heart, kidney and lower in small intestine than in WT males (Fig. 2d, f). The expression of the truncated ACE2 was also differentially affected by p53 deficiency, as most evidently shown by its absence in the small intestine of *flTP53^{R167H}* females (Fig. 2c and d).

4. Discussion

The sex-related COVID-19 mortality is the most frequently reported epidemiologic data [18]. The ACE2 is the main receptor of SARS-CoV-2 and its double-edged role as both an infection-promoting factor and a disease-protective agent has been suggested [19]. So far, the gender-specific regulation of ACE2 is not fully understood.

In this study, we showed the tissue- and gender-specific regulation of *ACE2* by p53 in pigs. First, a wider expression of ACE2 in female than male pigs was found, which can be explained by hormonal regulation [20]. A positive effect of oestrogen on the ACE2 expression in men's myocardium was observed [21]. Besides, the expression of ACE2 increases with human age [4], along with changes in the concentrations of sex hormones.

Altogether, our study shows that the tissue-specific expression of ACE2 is similar in humans and pigs, which correlates with the evolutionary conservation of ACE2 structure between these species [10]. Notable, the highest ACE2 expression was found in the small intestine and kidney, a result consistent with data in humans [22].

Little is known about the role of p53 in the regulation of ACE2. An earlier study reported that p53 suppresses the replication of coronavirus through ACE2 degradation in humans [23] and inhibits the interferon-mediated antiviral immunity in pigs [24]. In this study, we show that the absence of the p53 binding site increases the activity of ACE2 promoter in porcine kidney cells. This data was confirmed by increased ACE2 expression in the kidney of *flTP53^{R167H}* pigs. We also found a tissue- and gender-specific effect of *TP53* knockout on the expression of the full length and truncated ACE2 isoforms.

5. Conclusions

The study provides important findings into gender-specific *ACE2* regulation by p53 in pigs. Considering the similarities of *TP53* function between pig and human [25], this work provides useful evidence for further studies in human patients.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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