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Pkd2 deletion during embryo development does not alter mesonephric programmed cell senescence

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ABSTRACT Programmed cell senescence during embryo development is a recently described process that opens a new perspective to understand the senescence response and that adds a new player whose contribution to development needs to be addressed. Identifying developmental syndromes with a root in deregulated programmed cell senescence will undoubtedly reinforce our view of senescence and could provide a new angle to confront disease. One of the structures that was initially reported to undergo cellular senescence is the mesonephros. During E12.5-E14.5, before regression, mesonephric tubules are positive for the most widely used marker of cell senescence, SAβG, and negative for proliferation marker, Ki67, in a p21Cip1-dependent manner. *PKD2* is one of the genes defective in autosomal dominant polycystic kidney disease (ADPKD). Inherited mutations in this gene result in cyst formation in adults after a secondary hit. Polycystin-2 (PC2) protein, the product of *PKD2* gene expression, inhibits cell cycle progression by inducing p21Cip1, whereas mutated *PKD2* results in increased proliferation and defective differentiation of kidney epithelial cells. Here, we addressed the possibility of defective programmed cell senescence as a consequence of *Pkd2* deletion in mice. We analyzed embryos for the expression of the senescence marker SAβG, for the proliferative status of mesonephric tubule cells, and for the expression of p21Cip1, without identifying any noticeable deregulation of cell senescence. Our results exclude defective programmed cell senescence upon *Pkd2* ablation as an initial event in ADPKD.

KEY WORDS: *Pkd2, cellular senescence, development, mesonephros*

Cell senescence has been considered traditionally a stress response triggered to stop proliferation of cells after exposure to potentially dangerous stimuli (Collado and Serrano, 2010). This cell protective response is also linked to cellular aging as a result of the time dependent accumulation of senescent damaged cells in tissues (Collado *et al.*, 2007). However, recently, programmed cell senescence was also described as part of the embryo development helping shape morphological structures by actively promoting cell population replacement in certain particular tissues and contributing to organ formation (Muñoz-Espín *et al.*, 2013; Storer *et al.*, 2013).

Among the different tissues and organs in which cell senescence has been identified, such as the endolymphatic sac of the inner ear, the closing of the neural tube, the vibrissae, or the apical ectodermal ridge, cell senescence is a contributing force for the kidney. The mesonephros is a transitory embryonic kidney acting before the maturation of the definitive kidney, or metanephros, and

Abbreviations used in this paper: ADPKD, autosomal dominant polycystic kidney disease; PC2, polycystin-2.

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essentially disappearing at around E15.5 (Davidson, 2008). Before that, and starting at around E12.5, mesonephric tubules undergo cellular senescence: they are positive for senescence-associated beta-galactosidase (SAβG) staining, the most widely used and accepted marker of cell senescence, and at the same time they are negative for the proliferation marker Ki67, and express cell cycle and senescence mediator p21Cip1 (the product of the *Cdkn1a* gene) (Muñoz-Espín *et al.*, 2013). All these markers disappear at around E15.5, a time when mesonephric tubules are already hardly detectable. Deficiency in senescence mediator p21Cip1 strongly reduces the activity of SAβG and rescues proliferation in the mesonephric tubules. However, the existence of redundant compensatory mechanisms based on apoptosis allows normal development to proceed, and senescence deficiency due to loss of p21Cip1 only results in an increased incidence of vaginal septa that compromises the fertility of *Cdkn1a*-null female mice (Muñoz-Espín *et al.*, 2013). Despite this relatively mild phenotype, defective senescence during embryo development is a plausible cause for syndromes with a developmental origin.

Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent monogenic human disorder and is characterized by bilateral renal tubular cyst formation and progressive enlargement (Gabow, 1993). ADPKD is associated with mutations mainly in *PKD1* (responsible of ADPKD-Type I), with 80–85% of ADPKD families carrying mutations for this gene, and in *PKD2* (-Type II), with 15–20% of patients carrying mutations in *PKD2* (Cornec-Le Gall *et al.*, 2017). Affected individuals carry germ line mutations in these genes, although cysts development does not occur until adulthood. Mouse models of *Pkd1* and *Pkd2* have been generated and, interestingly, complete deletion of *Pkd2* results on cysts formation as early as E15.5, implying the existence of defects preceding this developmental stage caused by the absence of *Pkd2* (Wu *et al.*, 1998). One of the effects described for deficiency of *Pkd2* is increased proliferation and branching morphogenesis in kidney epithelial cells (Grimm *et al.*, 2006) and, indeed the overexpression of Polycystin-2 (PC2) protein encoded by *Pkd2* leads to a cell growth arrest that is mediated by inhibition of Id2 transcription factor (Li *et al.*, 2005). Id2 is a positive cell cycle regulator that accumulates in the nucleus to promote proliferation and to regulate differentiation at least in part by suppressing p21 induction. It has been reported that PC2 modulates Id2 by directly binding to Id2 in a PC1-dependent manner (Li *et al.*, 2005). In agreement with these observations, expression of both PC1 and PC2 reduces the cell cycle by blocking Id2 action over p21, and it has been described that PC1 overexpression induces p21 levels in MDCK cells in a PC2-dependent manner (Bhunia *et al.*, 2002).

With all these previous knowledge, we decided to investigate whether *Pkd2* deletion during embryo development in mice could result in a defective cellular senescence response in the mesonephros that could be the origin of cysts formation later on in the adult.

Results

In order to analyze the cell senescence response in *Pkd2* deficient embryos we set up crosses of *Pkd2* heterozygous animals. In this manner, we obtained littermate embryos of *wild type* (WT) and *Pkd2*-null genotypes. We checked vaginal plugs the following day and estimated the gestational period of time. At E13.5 we sacrificed the pregnant mice, extracted the embryos, fixed them immediately in fixation solution, and whole mount-stained them by incubation in SAβG staining solution overnight at 37°C. Direct macroscopic inspection of stained *Pkd2*-null embryos showed positive reaction in the apical ectodermal ridge of the limbs, the closing of the neural tube or the vibrissae with similar intensity than the one observed for WT embryos (Fig. 1).

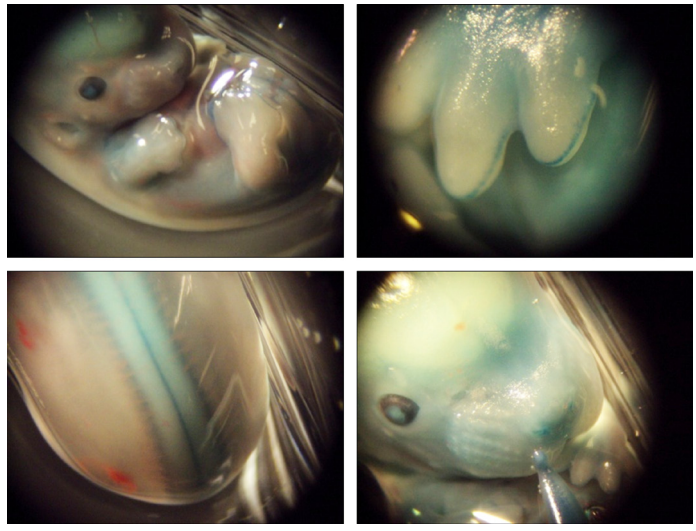

Fig. 1. Representative pictures of *Pkd2*-null embryos stained with SAβG. *Photographs under magnifying glass of a stained whole embryo, the fore limb showing the apical ectodermal ridge, the closing neural tube and vibrissae (from top to bottom and left to right).*

After staining, embryos were washed, dessicated in ethanol and embedded in paraffin. We sectioned these embryos and checked for the appearance of SAβG stained areas in the embryos, corresponding to the endolymphatic sac and mesonephric tubules, as reported (Muñoz-Espín *et al.*, 2013). Consecutive sections spanning the stained structures were further used for immunohistochemical analysis using antibodies specific for a proliferation marker, Ki67, and for the cell senescence and cell cycle inhibitor marker, p21Cip1. Sections from WT embryos showed a strong SAβG blue positive staining in the endolymphatic sac (Fig. 2A), a structure that is part of the inner ear and that has been described to present a strong SAβG staining at this time during development. The epithelium of this structure was concomitantly mainly negative for the proliferative Ki67 marker and positive for the cell senescence marker p21Cip1, validating our analysis. In the case of the *Pkd2*-null embryos we observed the exact same pattern, implying a normal cell senescence response in these embryos for this specific structure (Fig. 2A).

Next, we focused our attention on the mesonephros. This is an elongated structure, adjacent to the gonads, and longitudinally crossed by the mesonephric or Wolffian ducts, from which numerous lateral tubules emerge (Sainio, 2003; Vazquez *et al.*, 1998). In contrast to the endolymphatic sac, the WT mesonephric tubules appear very weakly positive after whole-mount SAβG staining with pale and punctuated blue dots surrounding the cells that are part of the tubules (Fig. 2B). These tubules were mainly negative for the proliferation marker, Ki67, and showed positive staining for p21Cip1 senescence marker, as reported previously. When *Pkd2*-null embryos were analyzed we observed the exact same pattern, with light but clear SAβG staining, absence of proliferation

as judged by lack of Ki67 staining, and positive p21Cip1 staining, suggesting a normal cell senescence response during embryo development of the mesonephros (Fig. 2B). We speculated with the possibility that a putative defective senescence could be observed at a later time point. For this, we repeated this same analysis in embryos at E14.5 obtaining essentially the same results (Fig. 2 A,B), confirming that cellular senescence remained unaltered by deficiency of *Pkd2* in the mouse.

Discussion

The recent description of senescence contributing to embryo development highlights the relevance of this crucial cellular response and offers another example of the parallelism between this process and apoptosis, both terminal cellular responses that can be triggered either by a stress response or upon developmental cues (Muñoz-Espín and Serrano, 2014; Pérez-Garijo and Steller, 2014). At this early stage, we can only speculate on the role played by cellular senescence during development. A powerful experimental approach to reveal the function of cellular senescence would be to cancel the process on animal models and observe the effect produced by its absence on the adult animal. Another alternative would be to identify a pathology associated with altered senescence during development. Given that ADPKD is a two-hit pathology originated already during development upon expression of a mutated *Pkd1* or *Pkd2* gene (Qian *et al.*, 1996; Watnick *et al.*, 1998), we wondered whether we could detect a deficient cellular senescence response in null embryos that could result in increased unscheduled proliferation and altered differentiation of the primitive embryonic kidney, and that could account for the defects that would eventually lead to the pathology in the adult.

Our global analysis of the senescence response did not show any altered pattern of SAβG staining, nor of the expression of the proliferative marker Ki67 or of the senescence mediator p21. When we inspected in detail the presence of these markers in the mesonephros at two different time points, E13.5 and E14.5, the time at which cellular senescence has been described to contribute to kidney development, we could not observe any defects in the senescence response, confirming that cellular senescence is not altered by the absence of *Pkd2*.

The delicate regulation of a crucial process such as development would probably imply that gross defects will not be tolerated and, at the same time, alteration of senescence could be counterbalanced by other mechanisms, such as increased apoptosis. Despite this

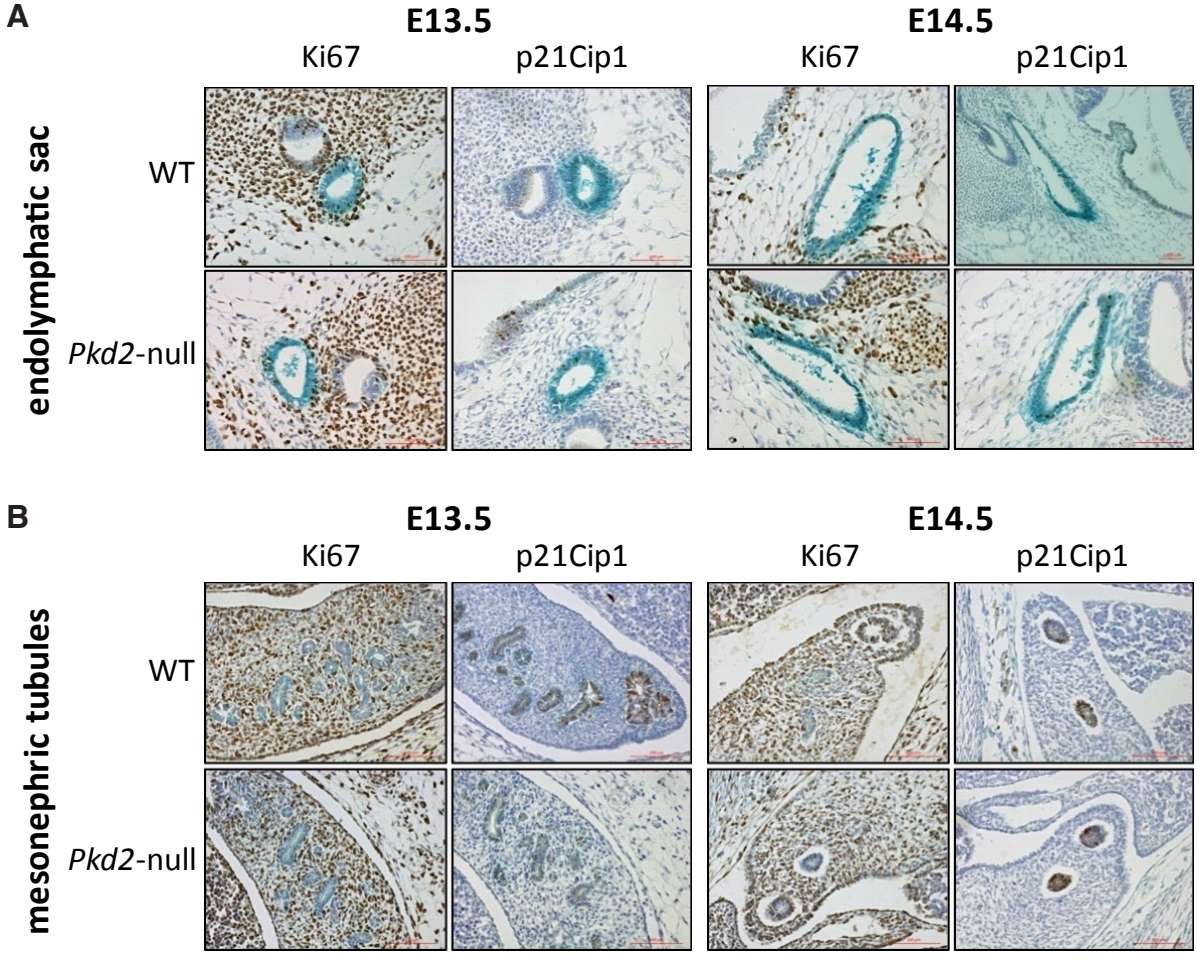

Fig. 2. Expression of SAβG, Ki67 and p21Cip1 in WT and *Pkd2*-null embryos. (A) *Sections of WT (top) and* Pkd2*-null (bottom) embryos previously stained with SAβG and marked for the expression of Ki67 (left) and p21Cip1 (right) in embryos of E13.5 (left panels) or E14.5 (right panels) showing the endolymphatic sac area.* **(B)** *Same analysis on areas showing the mesonephric tubules.*

result, and given the description of cellular senescence in embryos, alterations in this process during development will undoubtedly contribute to developmental syndromes. We need to carry on doing further work to identify developmental syndromes associated with defects in cellular senescence to better understand the bases of these pathologies and to try to develop appropriate therapies.

Materials and Methods

Mice

Pkd2 deficient embryos were obtained by crossing heterozygous mice (Wu *et al*., 1998) to obtain litters representing null and WT genotypes. Genotyping was done as published using DNA extracted from the yolk sacs. Embryos were re-genotyped using DNA extracted from paraffin blocks after histological and immunohistochemical analysis. Animals were kept under SPF conditions and all experiments were approved by the Santiago de Compostela University Bioethics Committee (protocol number 15005AE/07/01/02/05C/AVF2) in compliance with Principles of Laboratory Animal Care of national laws.

Senescence-associated beta-galactosidase staining

Whole mount Senescence-Associated beta-Galactosidase (SAβG) staining was performed on E13.5 or E14.5 embryos derived from crosses of *Pkd2* heterozygotic animals (a total of 4 WT and 3 null embryos). After extracting the uterine horns we separated each embryo from its placenta and embryonic sac and fixed them for 25 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde. After fixation, embryos were washed and incubated overnight at 37°C with fresh SAβG staining solution: 1 mg of 5-bromo-4-chloro-3-indolyl beta-D-galactoside (X-Gal) per mL (Fisher Scientific), 40 mM citric acid/sodium phosphate pH 5.5, 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, 150 mM NaCl, and 2 mM $MgCl_2$ (Dimri *et al*., 1995). Stained embryos were photographed on a magnifying glass, dehydrated in ethanol and embedded in paraffin. Blocks were serially sectioned at 5μm until the first blue stained section was observed. H&E staining was performed to confirm the presence of the desired organs (i.e. mesonephros and endolymphatic sac) and consecutive sections were obtained until blue staining disappeared for analysis.

Immunohistochemical analysis

SAβG stained sections were further used for immunohistochemical analysis with antibodies against proliferative marker Ki67 (prediluted SP6, Master Diagnostica 0003110QD) and senescence mediator p21Cip1 (HUGO-291 CNIO). The slides of interest were incubated overnight at 37°C before being deparaffinized in xylene and rehydrated in a descending series of ethanol solutions. Antigen retrieval was carried out in a PTLink instrument (Dako) and immunoreactive cells were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as a chromogen. Sections were counterstained with hematoxylin.

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Conflict of interest

Authors declare no conflict of interest.

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