CRISPR mediated somatic cell genome engineering in the chicken

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ABSTRACT

Gene-targeted knockout technologies are invaluable tools for understanding the functions of genes in vivo. CRISPR/Cas9 system of RNA-guided genome editing is revolutionizing genetics research in a wide spectrum of organisms. Here, we combined CRISPR with in vivo electroporation in the chicken embryo to efficiently target the transcription factor PAX7 in tissues of the developing embryo. This approach generated mosaic genetic mutations within a wild-type cellular background. This series of proof-of-principle experiments indicate that in vivo CRISPR-mediated cell genome engineering is an effective method to achieve gene loss-of-function in the tissues of the chicken embryo and it completes the growing genetic toolbox to study the molecular mechanisms regulating development in this important animal model.

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1. Introduction

Recent advances in the targeted modification of complex eukaryotic genomes have unlocked a new era of genome engineering. From the pioneering work using zinc-finger nucleases (ZFNs (Porteus and Carroll, 2005)) and transcription activator-like effector nucleases (TALEN (Miller et al., 2011)), to the recent development of the highly accessible clustered, regularly interspaced palindromic repeats (CRISPR)/Cas9 methodologies (Barrangou, 2014; Jinek et al., 2012; Pennisi, 2013; Wu et al., 2014), we now possess an unprecedented ability to analyze developmental processes using sophisticated designer genetic tools (Peng et al., 2014).

While CRISPR-mediated gene editing is widely used to generate loss-of-function in embryos and Primordial Germ Cells (PGCs) (Doudna and Charpentier, 2014; Hsu et al., 2014), this technology has also been used in mice to perform in vivo genome editing of somatic cells (Sánchez-Rivera et al., 2014; Wang et al., 2014; Xue et al., 2014; Yin et al., 2014). This approach creates genetic mutations in a subset of cells within a wild-type background, a technology that was used extensively in the Drosophila field to study complex biological processes (Blair, 2003). The electroporation technique, extensively used in the chicken embryo (Itasaki et al., 1999; Nakamura and Funahashi, 2013; Scaal et al., 2004; Serralbo et al., 2013; Voiculescu et al., 2008; Yokota et al., 2011), also results in the mosaic expression of constructs, which, combined to loss-of-function approaches, could provide similar advantages as in fly. However, gene inactivation in the chicken has been limited to knockdown by RNA interference- and morpholino-based methodologies (Das et al., 2006; Gros et al., 2009; Hou et al., 2011; Norris and Streit, 2014; Rios et al., 2011; Serralbo and Marcelle, 2014; Voiculescu et al., 2008) that each have their own limitations, including variability in the level of knockdown, off target effects, and transient inhibition of transcripts.

Here we show that CRISPR-mediated gene targeting is an amenable method of in vivo somatic cell genome editing in the chicken. This technology will allow refining the spatial and temporal roles of genes during embryonic development. Additionally this technology opens the door to further advances in the genetic manipulations of avian species. Indeed, recent reports of genetically modified chickens using targeted gene knockout using the TALEN technology in isolated chicken Primordial Germ Cells (PGCs) (Park et al., 2014) suggests that the same strategy combined with the easier CRISPR technique may soon be used at a large scale to generate specific genome-edited avian lines.

2. Results and discussion

2.1. Design of an inducible CRISPR-mediated gene-targeting system

We examined whether the CRISPR mediated gene-targeting methodologies could be combined with the in ovo electroporation technique for loss-of-function experiments in the chicken embryo. As a proof-of-principle, we targeted the transcription factor PAX7,
strongly expressed in the dorsal compartment of somites (the dermomyotome) and in the dorsal part of the neural tube in early amniote embryos. Since most if not all dermomyotome and dorsal neural tube cells normally express this gene, we reasoned that an effective loss-of-function of PAX7 would be unequivocally detected (using a highly specific antibody against Pax7) as a lack of PAX7 immunostaining in electroporated cells.

Our experimental setup necessitated that we have a robust strategy that results in a conspicuous loss of the PAX7 protein. The strategy of using two guide RNAs (gRNAs) to delete an intervening segment by the introduction of two Double Stranded Breaks (DSB), with repair via non-homologous end joining (NHEJ) has been shown to be the most efficient way to generate a defined genomic deletion (Canver et al., 2014; Ran et al., 2013; Zhou et al., 2014). We therefore designed several pairs of gRNAs targeting exon 1 and exon 2 of Pax7 (Fig. 1A). By using various combinations of these gRNAs, we expected to generate deletions ranging from 28 bp to over 2 kb in length. Such deletions should result in frame shift mutations, premature stop codons and truncation of the majority of the PAX7 protein (but for the N-terminal 24–33 amino acids, depending on the gRNA pairs; Fig. 1A).

As in ovo electroporation results in a mosaic population of transfected cells, we designed an experimental system that would allow the identification of CRISPR-targeted cells within the electroporated tissue. Also we required a system that was amenable to long-term analyses and be inducible, so that we could activate the CRISPR-mediated deletion at different stages of embryogenesis. We therefore designed an inducible CRISPR vector strategy, which combines features from the Tet-On Advanced system (Clontech), Cas9 and gRNA vectors (Mali et al., 2013a) with the Tol2 transposable elements (Sato et al., 2007; Serralbo et al., 2013; Sieiro-Mosti et al., 2014; Yokota et al., 2011).

The first vector contains the Tet-On transactivator under the control of the ubiquitous CAGGS promoter (CAGGS-rtTA). The second vector contains a bi-directional tetracycline-response element (TRE), which in the presence of doxycycline and rtTA, drives the simultaneous expression of membranal EGFP and mammalian codon-optimized, nuclear localized Cas9 (TRE-Cas9-GFP). The third vector contains one Pax7-specific gRNA driven by the human U6 promoter, as well as a second cassette containing the CAGGS promoter driving RFP (U6-gRNA-RFP). Additionally, as all vectors contain flanking sequences from the Tol2 transposable element, this allows their integration into the genome of electroporated cells via the Tol2 transposase, thereby avoiding the gradual dilution of plasmids with cell division. The transposase, driven by a CAGGS promoter, is provided on a fourth vector (CAGGS-Transposase). This combination of 5 vectors (CAGGS-rtTA; TRE-Cas9-GFP; 2X U6-gRNA-RFP; CAGGS-Transposase; illustrated in Fig. 1B) permits the inducible expression of Cas9, and the identification of CRISPR Pax7-targeted cells as GFP- and RFP-positive cells.

2.2. CRISPR mediated deletion of PAX7

The Pax7-specific gRNA pairs along with the Tol2 flanked, inducible, CRISPR mediated gene-targeting vectors were electroporated into the dorsal neural tube or the newly formed somites of E2.5 chicken embryos. We examined the loss of PAX7 in CRISPR targeted cells 24 h post-electroporation, by immunofluorescence
using the PAX7 monoclonal antibody. In addition, we performed PCR-based mutation analysis to characterize the corresponding deletion. At E3.5, PAX7 is highly expressed in the dorsal-most portion of the neuroepithelium (Jostes et al., 1990; Marcelle et al., 1995). Electroporation of the Pax7-gRNA pair (1.1 and 1.7) into the neural tube along with the CRISPR mediated gene-targeting vectors resulted in a significant decrease or a loss in PAX7 staining in Cas9/eGFP and gRNA/RFP positive cells, when compared to control cells within the neural tube (Fig 2A–C). Quantitative analysis of the PAX7 staining showed a robust decrease of PAX7 expression, since only 20.95% of electroporated cells displayed normal levels of PAX7 staining when compared to control electroporated cells, suggesting that PAX7 knockdown has occurred in approximately 80% of cells (Fig 2G). As controls, we verified that this procedure did not affect the expression of the closely related transcription factor PAX3, or of the acetylated form of Tubulin. In addition, DAPI staining confirmed that it did not lead to any visible increase in apoptosis (Supplementary Fig. 1).

PAX7 gene knockdown was also performed in the DML of somites. Initially homogenously expressed in the dermomyotome, Pax7 expression becomes progressively weaker in differentiating somites. Initially homogenously expressed in the dermomyotome, Pax7 staining con

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Fig. 2. The neural tube or newly formed somites in E2.5 chicken embryos were electroporated with the Tol2 flanked, inducible, CRISPR mediated gene-targeting vectors, with doxycycline added at the time of the electroporation. (A–C) Transverse sections of the neural tube or (D–F) confocal slice through the DML of a somite, 24 h post-electroporation demonstrating the loss of Pax7 immunostaining specifically in the GFP and RFP labeled cells. Graphs illustrating the percentage of cells expressing high levels of Pax7 in the (G) neural tube or (H) DML of cells electroporated with Pax7 targeted gRNAs versus control (i.e. no gRNA) electroporations. *** denotes P value of ≤ 0.001. Scale bars: 25 μm.
clones (Fig. 4). Only 1 of 18 clones from Pax7 gRNA pair 1.7 and 2.16, and 1 of 14 clones from 1.7 and 2.17 contained the exact predicted deletion, while the majority of cloned sequences contained much larger deletions, with up to an additional 341 bp of genomic sequence deleted in one instance, although on average 130 bp of sequence were deleted. This is surprising, as previous reports of CRISPR mediated gene targeting in Xenopus, Zebrafish and Drosophila embryos suggest that the NHEJ repair mechanism is very precise (Gratz et al., 2014; Guo et al., 2014; Hruscha et al., 2013; Irion et al., 2014; Shen et al., 2014). However, our results would suggest that the process of NHEJ can be error prone, maybe in the specific context of electroporation. Importantly, regardless of the range in size of the Pax7 genomic deletions, sequence analysis of the CRISPR targeted clones confirmed that all these deletions resulted in aberrant mRNA splicing, changes to the open reading frame and the presence of premature stop codons that would result in truncated Pax7 protein, thereby explaining the loss of Pax7 staining, detected by immunofluorescence in these cells.

3. Conclusion

This series of proof-of-principle experiments indicate that in vivo CRISPR-mediated cell genome engineering is a highly effective method to achieve genetic mutations in a subset of cells of the chicken embryo. Future improvements to the technology described here will aim to reduce the number of co-electroporated vectors: we show here that the co-electroporation of five vectors is efficient. However, this may become a limiting factor if an alternative strategy using modified Cas9 “nickase” is utilized. While the main advantage of the Cas9 nickase is to greatly reduce the off-target activity of the Cas9 endonuclease, it utilizes paired gRNAs to create targeted double strand breaks in the genome (Mali et al., 2013a; Ran et al., 2013). Large deletions described here would then require four gRNAs (i.e. four U6-gRNA-RFP vectors). Multiplex gRNA cloning kits that gather two or even four gRNAs at once in a single vector (Systembio) would very effectively reduce this number. Despite, this advancement in gene engineering now...
permits large scale loss-of-function analysis of candidate genes in the chicken and the entry of this important animal model in the genetic era.

4. Materials and methods

4.1. CRISPR mediated gene-targeting vectors

The Tol2-pTRE-Bl-eGFPcaax vector was derived from the bi-directional doxycycline inducible pTRE-Bl-eGFP vector (Clontech) by the replacement of eGFP with a membrane-localized eGFP (containing the CAAX prenylation sequence from H-Ras) along with the addition of flanking Tol2 integration sequences, into which nuclear localized, human codon optimized Cas9 (Addgene #41815, (Mali et al., 2013b)) was cloned to generate the TRE-Cas9-eGFP. We previously showed that this inducible plasmid system is silent without doxycyclin (Rios et al., 2011). Tol2-CAGGS-rtTA has been described previously (Serralbo et al., 2013). The hU6-gRNA empty vector was obtained from Addgene (#41824, (Mali et al., 2013b)). The hU6-gRNA scaffold cassette was cloned into Tol2-CAGGS-RFP (Serralbo and Marcelle, 2014) to generate the U6-gRNA-RFP. Pax7 target gRNAs were designed and selected as described in Mali et al. (2013b), with further verification of specificity and off target effects using the CRISPR design tool (CRISPR.mit.edu, (Hsu et al., 2014)). The verified gRNA target sequences (Table 1) were cloned into the hU6 promoter gRNA scaffold primers in the hU6 gRNA vector as described (Mali et al., 2013b). An empty gRNA vector was used for control electroporations.

4.2. In ovo electroporation and imaging

Fertilized chicken eggs were incubated at 38 °C in a humidified incubator. Embryos were staged according to days of incubation.
and number of somites. The neural tube and somites were electroporated as previously described (Gros et al., 2004; Rios et al., 2011). The final concentration for each plasmid in the electroporation mix was 1 μg/ml. Doxycycline (300 μl of 15 μg/ml) was added at the time of electroporation. Embryos were analyzed under UV examination 24 h after electroporation and correctly electroporated embryos (i.e. high expression of the fluorescent reporters and no visible malformation due to electroporation) were dissected and fixed for 1 h in 4% formaldehyde.

Embryos were either analyzed by whole mount immunostaining or embedded in 15% sucrose/7.5% gelatin/PBS solution and sectioned into 20 μm slices using a cryostat (as described in Serralbo and Marcelle (2014)). Immunohistochemistry on sections or whole mount embryos was performed with the following antibodies: chicken polyclonal antibody against GFP (Abcam #ab13970), rabbit polyclonal anti RFP (Abcam #ab62341), mouse monoclonal Acetylated Tubulin (Sigma #T7451), mouse monoclonal anti Pax7 (Hybridoma Bank), mouse monoclonal anti Pax3 (Hybridoma Bank) detected with species-specific secondary antibodies coupled to AlexaFluor-488, 555, or -647 (LifeTechnologies). Whole-mount embryos and sections were imaged using a Leica SP5 confocal microscope running LAS AF software (Leica Microsystems).

Image stacks were analyzed by using either (i) the Imaris software package (Bitplane, version 7.5.2) or (ii) ImageJ software. Using the “spot” module of the Imaris, the region of interest was manually specified for each somite and an initial quality count for gRNA-containing cells was performed. Selected cells were then filtered based on central intensity of Pax7 staining, and the intensity peak value recorded as the percentage of positive cells. Manual cell counting was performed using the cell counter plugin (Kurt De Vos, University of Sheffield) within ImageJ (Schindelin et al., 2012) Statistical analyses were performed using the GraphPad Prism software. Student’s t-test was applied to populations to determine the P values indicated in the figures. In each graph, columns correspond to the mean and standard deviation. *** denotes P < 0.001.

4.3. PCR analysis

Twenty-four hours after electroporation, tissue expressing both GFP and RFP was dissected and pooled from three to five embryos and genomic DNA extracted. The CRISPR targeted region was amplified by a nested PCR approach with primers detailed in Table 2, that preferentially amplified the CRISPR deleted region by limiting the extension time of the PCR to 1 min. In the first round of PCR amplification of the wildtype band (with forward and reverse primers 107 and 106) would result in an ~3 kb band. A second round of PCR was then performed (with forward and reverse primers 74 and 106) which preferentially amplified the ~750 bp CRISPR deleted band over the ~2.9 kb wildtype band. The PCR products were gel purified and cloned into the pGEM-Easy vector (Promega) by TA cloning. Ten single colonies were randomly picked for DNA sequencing analysis to detect the insertion or deletion of bases. Sequences were aligned and analyzed using SnapGene (GSL Biotech).

Table 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>74 Pax7 gDNA F1</td>
<td>ctcgcggcgcctggctatgg</td>
</tr>
<tr>
<td>106 Pax7 gDNA R2</td>
<td>cggccttcgaagctgcttcccgg</td>
</tr>
<tr>
<td>107 Pax7 gDNA F2</td>
<td>ggtgtaggagaccttcgcagaacc</td>
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Author contributions

CH and CM planned the experiments and wrote the manuscript, CH, NV and ZQ performed and analyzed the experiments. PK quantified the experiments and provided the corresponding graphs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.08.007.

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