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RESEARCH ARTICLE

Production of chimeras between the Chinese soft-shelled turtle and Peking duck through transfer of early blastoderm cells

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SUMMARY

Chimeras are useful models for studies of developmental biology and cell differentiation. Intraspecies and interspecies germline chimeras have been produced in previous studies, but the feasibility of producing chimeras between animals of two different classes remains unclear. To address this issue, we attempted to produce chimeras between the Chinese soft-shelled turtle and the Peking duck by transferring stage X blastoderm cells to recipient embryos. We then examined the survival and development of the PKH26-labeled donor cells in the heterologous embryos. At early embryonic stages, both turtle and duck donor cells that were labeled with PKH26 were readily observed in the brain, neural tube, heart and gonads of the respective recipient embryos. Movement of turtle donor-derived cells was observed in the duck host embryos after 48 h of incubation. Although none of the hatchlings presented a chimeric phenotype, duck donor-derived cells were detected in a variety of organs in the hatchling turtles, particularly in the gonads. Moreover, in the hatched turtles, mRNA expression of tissue-specific duck genes *MEF2a* and *MEF2c* was detected in many tissues, including the muscle, heart, small and large intestines, stomach and kidney. Similarly, *SPAG6* mRNA was detected in a subset of turtle tissues, including the gonad and the small and large intestines. These results suggest that duck donor-derived cells can survive and differentiate in recipient turtles; however, no turtle-derived cells were detected in the hatched ducks. Our findings indicate that chimeras can be produced between animals of two different classes.

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INTRODUCTION

A chimera is a complex individual that develops as a mixture of cells originating from two or more fertilized eggs belonging to the same or different species. Chimeras provide excellent models in which to study developmental biology and cell differentiation. Within a chimeric embryo, pluripotent donor cells can participate in the formation of recipient tissues and organs *in vivo* and differentiate accordingly. Moreover, donor primordial germ cells (PGCs) or precursors can differentiate into functional gametes in chimeric gonads. As the avian embryo is surrounded by a shell and develops *in vitro*, researchers can operate directly on the embryo. As a result, birds are often used to produce various types of somatic or germline chimeras (Douarin and McLaren, 1984).

In birds, germline chimeras can be made by transferring blastodermal cells or PGCs into recipients (Thoraval et al., 1994). Although it is more efficient to obtain germline chimeras by transferring PGCs, transferring blastodermal cells is much simpler. The pluripotent stage X blastoderm comprises 40,000–80,000 cells (Eyal-Giladi and Kochav, 1976) and contains precursors of both somatic cells and PGCs. When injected into a recipient embryo, a subset of the blastodermal cells contributes to somatic and germinal tissues (Thoraval et al., 1994). We therefore chose this method to produce chimeras in the present study.

Intraspecies germline chimeras have been produced between different strains of chickens (Petitte et al., 1990; Tajima et al., 1993; Kang et al., 2008; Kang et al., 2009) and quails (Kim et al., 2005). The successful production of interspecies germline chimeras, e.g. between quail and chicken (Naito et al., 1991; Ono et al., 1996), Maya duck and chicken (Li et al., 2002), Peking duck and quail (Sha et al., 2008; Gao et al., 2011) or Houbara bustard and chicken (Wernery et al., 2010), has also been reported. However, the production of chimeras between organisms belonging to two different classes has not been reported. To determine whether this is possible, we attempted to produce chimeras between the Peking duck (*Anas platyrhynchos* Linnaeus 1758) and the Chinese soft-shelled turtle (*Pelodiscus sinensis* Wiegmann 1835).

The Chinese soft-shelled turtle is an ancient, subaquatic reptile that occupies a unique place in the animal kingdom. This turtle has been used as a model of reptilian development, and recent molecular phylogenetic analyses have revealed its close affinity to birds, crocodiles and other turtles (Hedges and Poling, 1999; Kumazawa and Nishida, 1999; Mannen and Li, 1999; Cao et al., 2000; Zardoya and Meyer, 2001). Turtles and birds also share many similarities during early development. Embryos from both of these classes undergo discoidal meroblastic cleavage, wherein the early cell divisions do not cut through the yolk of the egg. These cells form the blastoderm (Gilbert, 2010).

We attempted to produce interspecies chimeras between the Chinese soft-shelled turtle and the Peking duck by transferring blastoderm cells into heterologous recipient embryos. We then

Table 1. Primers used for PCR, RT-PCR and qPCR analyses

Gene	Direction	Sequence	Product (bp)
CYTB[a]	Forward	GCACTACACCGCAGACAC	378
	Reverse	AAGAATCGGGTTAGGGTT	
CYTB[b]	Forward	ACAACCCAACAGGACTTA	243
	Reverse	TGTTAGGGATAGATCGTA	
12S rRNA[a]	Forward	AAGATGCCTATCCTACCTAA	534
	Reverse	GCGAGCTTTATTGTCCCGC	
12S rRNA[a,c]	Forward	AAGATGCCTATCCTACCTAAC	191
	Reverse	CTTCGGTAGGGTAAGTATCAAGA	
MEF2a[a]	Forward	GGTCCCAGTGTCCAATC	325
	Reverse	AGGCATGACTTTCCCTAA	
MEF2c[a]	Forward	TGAGTCTGAGGACAAGTACAGG	480
	Reverse	TTCACTGATGGCATTGTAT	
SPAG[a]	Forward	TTGGGCACTCGGGTAT	581
	Reverse	GTGGTGGTATTCCCTTG	
Ghrelin[a]	Forward	TGCTAACCCATCGGCTCCAT	295
	Reverse	CGTTGCCATCTTCTGTCCCT	
GAPDH[a]	Forward	TGCCATCACAGCCACACAGAAG	123
	Reverse	ACTTTTCCCACAGCCTTAGCAG	
GAPDH[b]	Forward	TGCCATCACAGCCACGCAGAAG	123
	Reverse	ACCTTGCCCACAGCCTTAGCAG	

[a]Duck-specific marker.
[b]Turtle-specific marker.
[c]Primers used for qPCR analyses.

examined whether the exogenous donor cells were able to survive and develop in the host embryos. At early embryonic stages, donor cells from both the turtle and the duck were easily detected in the respective recipients. However, by hatching, only duck donor-derived cells could be detected in the turtle hosts; these cells were found in a variety of organs, including the gonads. In these turtle hatchlings, mRNA expression of the duck tissue-specific genes *MEF2a* and *MEF2c* was detected in many tissues, including the muscle, heart, small and large intestines, stomach and kidney. *SPAG6* mRNA was also detected in certain tissues, including the gonads and the small and large intestines. These results indicate that duck donor-derived cells can survive and differentiate in recipient turtles. Our successful production of Peking duck–Chinese soft-shelled turtle chimeras thus suggests that it is indeed possible to generate chimeras between animals of two different classes.

MATERIALS AND METHODS

Embryos

Fertilized Chinese soft-shelled turtle eggs were purchased from the Maotian farm in Hebei Province, China, in July and August 2010. The eggs were incubated at 31°C and 85–95% relative humidity. The embryonic development of the eggs was measured in days of incubation to monitor the developmental stage. All of the animal care and handling procedures were performed in accordance with the standards of China Agricultural University.

Production of chimeras between the Chinese soft-shelled turtle and the Peking duck

Preparation of donor blastoderm cells

Blastoderms of Peking duck eggs and Chinese soft-shelled turtle eggs were isolated with filter paper rings at room temperature. The blastoderms were washed with phosphate buffered saline (PBS) to remove the adhering yolk and then transferred into 1.5 ml Eppendorf tubes. Each cell pellet was softly dispersed in PBS using a pipette; following dissociation, the cells were centrifuged at 1300 ***g*** for 3 min. The cells were then washed three times and resuspended in PBS. A portion of the cells were stained with 5×10^{-6} mol l^{-1} PKH26 fluorescent dye (Phanos Technologies, Japan) at 37°C for 10 min. The staining reaction was then stopped by adding serum for 1 min at 37°C (Hyclone, Logan, UT, USA), according to the manufacturer's instructions. The labeled cells were washed three times with PBS and counted using both light and fluorescence microscopy (Olympus, Narishige, Japan). The labeled blastodermal cells were resuspended at a concentration of 2000 to 2500 cells μl^{-1} in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) prior to microinjection. These cells were used to observe the distribution of donor cells at early embryonic stages. Unlabeled cells were also resuspended at a concentration of 2000 to 2500 cells μl^{-1} in DMEM and injected into recipients.

Injection into recipient embryos

Freshly laid Peking duck eggs and Chinese soft-shelled turtle eggs were swabbed with 70% alcohol, and a ~4 mm-diameter round window was made in the equatorial plane of each eggshell using forceps. Approximately 2000 to 3000 donor cells in 1 to 1.5 μl of PBS were transferred into the subgerminal cavity of the recipient blastoderm using a 60 to 80 μm glass micropipette, and the window in the eggshell was sealed with a small piece of Parafilm. The duck eggs were placed in an incubator maintained at 37.8°C and 65% relative humidity until hatching. The turtle eggs were placed in an incubator maintained at 31.0°C and 85–95% relative humidity until hatching.

Detection of fluorescent cells

Early embryos at different developmental stages (e.g. 48 h, 60 h, 3 days, 4 days and 7 days) were removed from the yolk and washed with PBS. The distribution of the PKH26-labeled donor blastoderm cells was noted, and the movement of the PKH26-labeled cells was recorded using video and fluorescence microscopy (Olympus). Successive pictures of moving cells were obtained from the video at different time points. The gonads of 7-day-old duck embryos were isolated, digested with trypsin and observed by fluorescence microscopy. The development of turtle embryos is much slower than that of duck embryos. As it is very difficult to isolate gonads from early turtle embryos, these organs were not analyzed at early stages.

Table 2. Hatching rates of chimeras between the Chinese soft-shelled turtle and the Peking duck

Donor	Recipient	Experimental group	No. of operated embryos	No. of hatchlings	Hatching rate (%)	No. of positive chimeras	Positive chimera rate (%)
Turtle	Duck	1	40	8	20.0 (8/40)		
		2	42	13	31.0 (13/42)		
		Total	82	21	25.6 (21/82)	0	0
Duck	Turtle	3	119	21	17.6 (15/85)		
		4	145	23	15.9 (23/145)		
		Total	264	44	16.7 (44/264)	5	11.4 (5/44)

Detection of donor cells in the chimeras using PCR, quantitative real-time PCR and RT-PCR

Genomic DNA was extracted from various tissues and organs of the chimeras by overnight digestion with proteinase K (Sigma-Aldrich, St Louis, MO, USA) followed by phenol extraction and ethanol precipitation (Taberlet and Bouvet, 1991). Duck- and turtle-specific primers were designed based on the sequence differences between the respective cytochrome *b* (*CYTB*; GenBank accession numbers: turtle, AY583692; duck, EU585609) and 12S rRNA genes (*12S rRNA*; GenBank accession numbers: turtle, AY743420; duck, U59666) of these species. The duck and turtle *GAPDH* genes (GenBank accession numbers GU564233.1 and AB124567.1, respectively) were used as reference genes. The chimerism rate of positive tissues as determined by genomic PCR was further quantified using absolute quantitative real-time PCR (qPCR). The primers used for qPCR were designed based on the sequence differences between *12S rRNA* genes in ducks and turtles. qPCR was performed using the LightCycler 480 system and the LightCycler 480 SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany). Three independent experiments were performed in triplicate, and the average tissue chimerism rate was determined. To further investigate the proliferation and differentiation of the donor cells, total RNA was extracted from turtle blastodermal cells, duck blastodermal cells and chimeric tissues, and was purified using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). One microgram of total RNA was reverse-transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI, USA), and the cDNA was used as the template for the following PCR. The primers used for reverse transcription PCR (RT-PCR) were designed based on the sequences of the duck myogenic enhancer factor 2a (*MEF2a*; GenBank accession number: HM460752), myogenic enhancer factor 2c (*MEF2c*; GenBank accession number: JX042310), sperm associated antigen 6 (*SPAG6*; GenBank accession number: EF575595.1) and ghrelin genes (*Ghrelin*; GenBank accession number: EF613551.2). The primers used for RT-PCR spanned a minimum of one intron. The PCR amplifications were conducted in a total volume of 25 µl. The resulting DNA fragments were separated on 1.2% agarose gels, and the bands were visualized by ethidium bromide staining. The sequences of the primers used for the PCR, qPCR and RT-PCR analyses are listed in Table 1.

RESULTS

Development of Chinese soft-shelled turtle embryos during the incubation period

A total of 98 turtle eggs were incubated at 31°C and 85–95% relative humidity for 40–42 days. Of these eggs, 54 survived to hatching, yielding an average hatching rate of 55.1% (54/98).

Production of chimeras between the Chinese soft-shelled turtle and the Peking duck

A total of 82 duck recipient embryos were injected with turtle blastodermal cells. Of these embryos, 21 survived to hatching, yielding an average hatching rate of 25.6% (21/82). A total of 264 turtle recipient embryos were injected with duck blastodermal cells. Of these embryos, 44 survived to hatching, yielding an average hatching rate of 16.7% (44/264) (Table 2). Although phenotypic chimerism was not observed in any of the embryos or hatchlings, duck-derived cells were detected in five turtle hatchlings, yielding a rate of positive duck–turtle chimerism of 11.4% (5/44). However, no turtle cells were detected in any of the 21 duck hatchlings (Table 2).

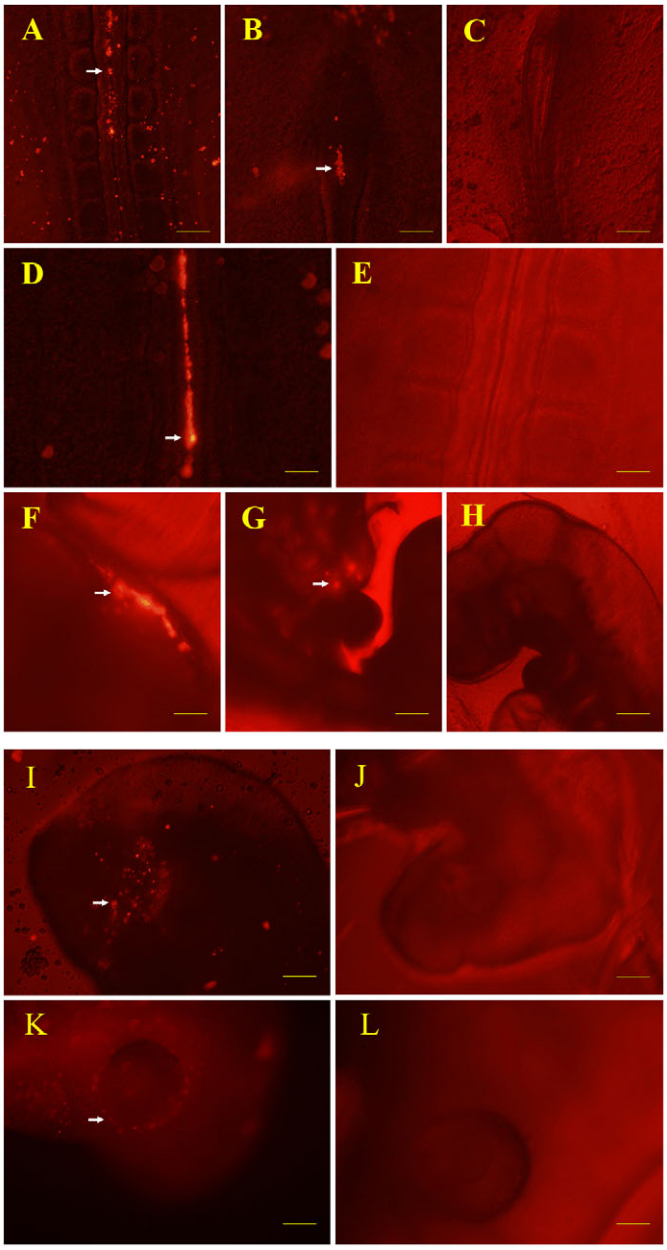

Fig. 1. Distribution of PKH26-labeled donor blastodermal cells in early recipient embryos. (A–H) Duck embryos. Fluorescent turtle cells were detected in the central part (A) and the end (B) of the duck neural tube after 48 h of incubation, the central part (D) of the duck neural tube after 60 h of incubation and the brain vesicle (F) and heart (G) after 3 days of incubation. (C) The central part and end of an uninjected duck neural tube after 48 h of incubation. (E) The central part of an uninjected duck neural tube after 60 h of incubation. (H) The heart and brain vesicle of an uninjected duck embryo after 3 days of incubation. Arrows indicate the PKH26-labeled donor cells. Scale bars: (A,B,F) 200 µm; (C,G,H) 500 µm; (D,E) 100 µm. (I–L) Turtle embryos. Fluorescent duck cells were detected in the brain vesicle of a turtle embryo after 4 days of incubation (I) and the eye after 7 days of incubation (K). (J) The brain vesicle of an uninjected turtle embryo after 4 days of incubation. (L) The eye of an uninjected turtle embryo after 7 days of incubation. Arrows indicate the PKH26-labeled donor cells. Scale bars: 200 µm.

Fluorescence observation

To monitor the distribution of donor cells at early embryonic stages, the cells were labeled with PKH26 prior to injection into the subgerminal cavity of recipient blastoderms. PKH26-labeled turtle

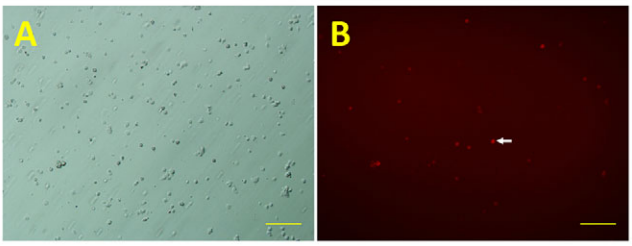

Fig. 2. Trypsinized gonadal cells of a turtle-duck chimera at 7 days of incubation. PKH26-labeled donor cells were detected in a Chinese soft-shelled turtle–Peking duck chimeric gonad after 7 days of incubation. (A) Brightfield image; (B) dark-field image. Arrow indicates a PKH26-labeled donor cell. Scale bars: 100 µm.

cells were principally observed in the brain, heart and neural tube of duck embryos at early embryonic stages (Fig. 1A,B,D,F,G), whereas PKH26-labeled duck cells were principally observed in the brain and eye of turtle embryos (Fig. 1I,J). Numerous turtle-derived cells were also detected within the trypsinized gonads of 7-day-old duck embryos (Fig. 2). In addition, movement of PKH26-labeled turtle cells was observed in the blood vessels of a 48-h duck embryo (Fig. 3; supplementary material Movie 1). These data suggest that both turtle and duck cells were able to survive in the recipients at least at early stages.

Distribution analysis of donor cells in the chimeras

Due to the lack of phenotypic chimerism, DNA samples from different organs of the recipients were analyzed by PCR to ascertain the presence of donor-derived cells in the hatchlings. Species-specific primers were designed based on sequence differences between the turtle and duck *CYTB* and *12S rRNA* genes. As shown in Fig. 4, only the duck-specific PCR product was present when duck genomic DNA was used as the template, and only the turtle-specific PCR product was present when turtle genomic DNA was used as the template, suggesting that our experimental design was able to discriminate between cells of the two species. The PCR assay was sufficiently sensitive to detect the presence of ten turtle cells among 1 million duck cells or vice versa (Fig. 4A). We observed the duck-specific fragment in nearly all of the organs of the recipient chimeric turtles, including the heart, liver, small and large intestines, brain, stomach, pancreas, kidney, gonads and muscles, indicating that the duck cells can survive in turtle hatchlings (Fig. 4B). However, the turtle-derived fragment was not detected in hatched recipient ducks (Fig. 4C). qPCR was then performed to further determine the chimerism rate of the chimeras. As shown in Table 3, the chimerism rate was very low, ranging from 0.0150 to 0.0788‰ in different tissues. The low chimerism rate may be the reason for the lack of phenotypic chimeras.

The presence of duck cells in different organs of the recipient turtles suggests that duck cells may differentiate into different tissues in a turtle host (Fig. 4B). To test this hypothesis, we examined whether four tissue-specific duck genes were expressed in the chimeras. MEF2a and MEF2c are myogenic enhancer factors that are specifically expressed in muscle tissues (Liu et al., 2012; Sekiyama et al., 2012). As shown in Fig. 5A, expression of the duck *MEF2a* and *MEF2c* genes was detected in the muscle, heart, small and large intestines, stomach and kidney of ducks but not in duck blastodermal cells or any turtle tissues. In the chimeric recipient turtles, however, we observed expression of the duck-specific *MEF2a* and *MEF2c* in many tissues and organs of the recipient turtles, including muscle, heart, small and large intestines, stomach and kidney (Fig. 5B). Mammalian *SPAG6* encodes a sperm flagellar protein that is important for structural integrity and is the central apparatus that controls flagellar motility in the sperm tail. *SPAG6* is principally expressed in the testis but can also be detected in tissues containing cells bearing 9+2 motile cilia, e.g. the brain, lung, oviduct and uterus in mammals (Horowitz et al., 2005; Kiselak et al., 2010). We determined that the duck *SPAG6* gene was expressed at high levels in the gonads, brain, small and large intestines and kidney, all of which may contain cells bearing motile cilia. *SPAG6* was expressed at low levels in the stomach and liver and undetectable in the heart, muscle, duck blastodermal cells and all turtle tissues (Fig. 6A,B). Similar to the *MEF2a* and *MEF2c* results, we detected the duck-specific *SPAG6* fragment in certain tissues and organs of the recipient turtles, including the gonad and the small and large intestines (Fig. 6C). These data strongly suggest that duck blastodermal cells can differentiate into different cell types in turtle host chimeras. Moreover, ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, and ghrelin-containing cells are present in the stomach of all vertebrate species (Kojima and Kangawa, 2005). As shown in Fig. 6B, the duck *Ghrelin* gene was expressed strongly in the stomach but very weakly expressed in the brain, small intestine and gonads. The duck *Ghrelin* gene was not detected in the large intestine, kidney, liver, heart, muscle, duck blastodermal cells, or any turtle tissues. We were unable to detect the duck-specific ghrelin fragment in any tissue of the chimeric turtles (Fig. 6C).

DISCUSSION

Chimeras are useful models for studies of developmental biology and cell differentiation. Both intraspecies and interspecies germline chimeras have been successfully produced in previous studies, though it is very difficult to produce chimeras between different species, even at low efficiency. To our knowledge, the production of chimeras between animals of two different classes has not been reported. Here, we attempted to produce chimeras between Chinese

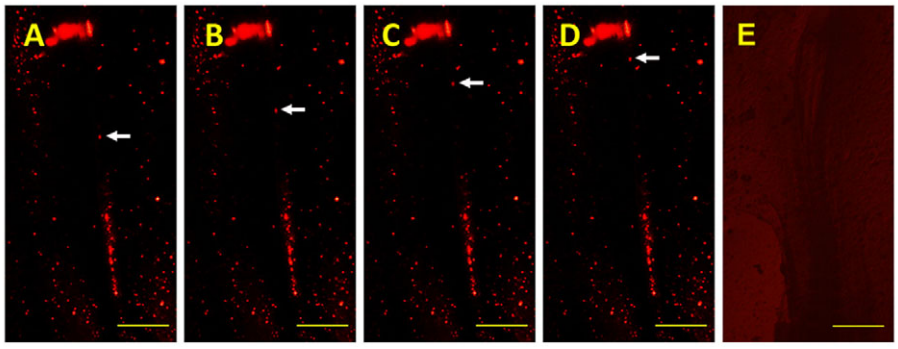

Fig. 3. Movement of turtle blastodermal cells in the blood vessels of early duck embryos. PKH26-labeled Chinese soft-shelled turtle blastodermal cells moved within the blood vessels of 48-h Peking duck embryos. (A–D) Successive pictures of donor cell movement at 0, 2, 4 and 5 s. (E) An uninjected 48-h Peking duck embryo. Arrows indicate the PKH26-labeled donor cells. Scale bars: 500 µm.

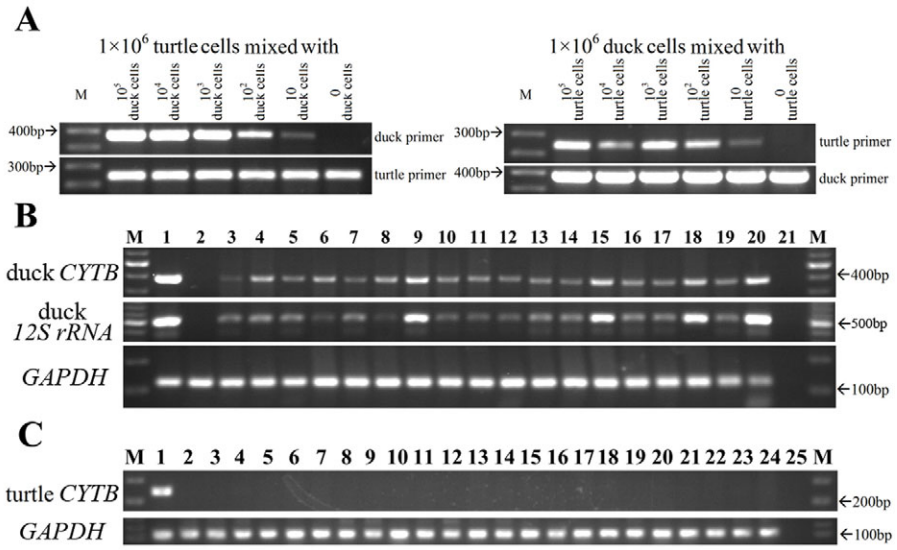

Fig. 4. PCR analysis of transferred donor cells in the chimeras. (A) Species identification sensitivity test: 1×10^6 turtle or duck cells mixed with decreasing quantities of duck or turtle cells. (B) PCR analysis of various tissues and organs of hatched duck–turtle chimeras using duck-specific *CYTB* and *12S rRNA* primers. M: Marker; Lane 1: positive duck blood; Lane 2: negative turtle blood; Lanes 3–9: heart, muscle, pancreas, kidney, large intestine, gonad and brain of a 1-yr-old female turtle; Lanes 10–19: heart, muscle, pancreas, liver, kidney, stomach, small intestine, large intestine, gonad and brain of a 1-yr-old male turtle; Lane 20: brain of a newly hatched turtle; Lane 21: blank control. (C) PCR analysis of various tissues and organs of hatched turtle–duck chimeras using turtle-specific primers. M: Marker; Lane 1: positive turtle blood; Lane 2: negative duck blood; Lanes 3–13: heart, muscle, pancreas, liver, kidney, proventriculus, gizzard, small intestine, large intestine, gonad and brain of a female duck; Lanes 14–24: heart, muscle, pancreas, liver, kidney, proventriculus, gizzard, small intestine, large intestine, gonad and brain of a male duck; Lane 25: blank control.

soft-shelled turtles and Peking ducks by transferring stage X blastoderm donor cells to recipients of the opposite species. At early embryonic stages, we observed donor cells from both turtle and duck in various host organs. At hatching, duck donor-derived cells were detected in a variety of organs of the host turtles, particularly in the gonads. While phenotypic chimeras were not observed, our results still suggest that it is possible to produce chimeras between animals of two different classes.

At early embryonic stages, we observed many turtle cells in various organs of the duck host, particularly the neural tube, brain and heart (Fig. 1A–H). Similarly, we observed many duck cells in different tissues and organs of early turtle embryos, especially the brain and eye (Fig. 1I–L). These results suggest that both turtle and duck cells can survive in heterologous host embryos. By the hatching stage, however, turtle donor cells could no longer be found in duck hosts, though duck-derived cells could still be detected in various tissues of the turtle recipients, including the heart, liver, small and large intestines, brain, stomach, pancreas, kidney, gonads and muscles (Fig. 4B). The results of the qPCR analysis indicated that the rate of tissue chimerism was extremely low (Table 3), ranging from 0.0150 to 0.0788‰. This fact may explain the lack of phenotypic chimerism.

It is very interesting that duck-derived cells were able to survive in the hatchling turtles, but turtle-derived cells could not survive in the duck. This discrepancy may arise from their evolutionary origins. The Peking duck, which belongs to the class Aves, evolved more recently than the turtle, which belongs to the class Reptilia. More research is required to test this idea. Another explanation for the observed difference in survival may lie in the different temperatures used for incubating the eggs.

Turtle cells were observed in the duck gonad (Fig. 2), suggesting that turtle PGC precursors may be able to incorporate into the blastoderms of the Chinese soft-shelled turtle embryos, which are similar to the avian embryos (Eyal-Giladi et al., 1981). We also observed turtle cells moving with the blood to the end of the neural tube, which is the site of the gonadal anlagen (Fig. 3), in 48-h duck host embryos. However, we are uncertain whether the movement of the cells was active or passive. If this movement represents an active migration, then turtle PGCs may be carried to the gonads *via* the blood and share the same migration patterns as chickens and snakes (Dubois, 1969; Nakamura et al., 2007; Bachvarova et al., 2009); however, more studies are required to test this idea.

Duck-derived cells appear to differentiate into many cell types in turtle host embryos. We observed that the duck tissue-specific genes *MEF2a* and *MEF2c* were expressed in many tissues and

Table 3. Chimerism rate of positive tissues as determined by PCR

Chimera	Tissue	Rate of tissue chimerism (‰)
1-yr-old female turtle	Heart	0.0150
	Muscle	0.0539
	Pancreas	0.0250
	Kidney	0.0466
	Large intestine	0.0229
	Gonad	0.0476
	Brain	0.0716
1-yr-old male turtle	Heart	0.0356
	Muscle	0.0396
	Pancreas	0.0281
	Liver	0.0341
	Kidney	0.0297
	Stomach	0.0618
	Small intestine	0.0398
	Large intestine	0.0301
	Gonad	0.0659
	Brain	0.0336
Newly hatched turtle	Brain	0.0788

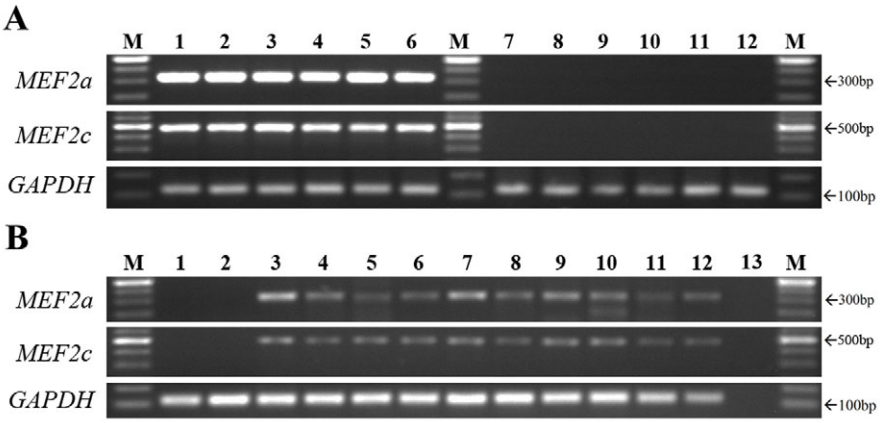

Fig. 5. RT-PCR analysis of various chimeric tissues and organs using duck tissue-specific *MEF2a* and *MEF2c* primers. (A) RT-PCR analysis of various tissues and organs of a duck and a turtle. M: Marker; Lanes 1–6: muscle, heart, stomach, small intestine, large intestine and kidney of a duck; Lanes 7–12: muscle, heart, stomach, small intestine, large intestine and kidney of a turtle. (B) RT-PCR analysis of duck blastodermal cells, turtle blastodermal cells and various tissues and organs of hatched duck-turtle chimeras. M: Marker; Lane 1: duck blastodermal cells; Lane 2: turtle blastodermal cells; Lanes 3–6: muscle, heart, large intestine and kidney of a 1-yr-old female turtle; Lanes 7–12: muscle, heart, stomach, small intestine, large intestine and kidney of a 1-yr-old male turtle; Lane 13: blank control.

organs of the hatchling turtles, including muscle, heart, small and large intestines, stomach and kidney (Fig. 5). Another duck tissue-specific gene, *SPAG6*, was also expressed in certain tissues and organs of the turtle hosts, including the gonad and small and large intestines, but not in tissues that do not express this gene in the duck; the same tissue distribution was observed for the expression of this gene in the duck (Fig. 6C). These results suggest that duck cells survived in the Chinese soft-shelled turtle and differentiated into various cell types. Although transferred blastoderm cells have been shown to differentiate into ectoderm, mesoderm and endoderm in early quail–chicken chimeric embryos (Watanabe et al., 1992), quail and chicken are much more closely related than ducks and turtles. It is therefore more surprising that duck blastoderm cells can differentiate and contribute to the organs of the turtle host.

Expression of the duck *Ghrelin* gene was not detected in the chimeric tissues, even in the stomach, where *Ghrelin* is highly expressed in the duck (Fig. 6C). There are at least three possible explanations for this result. The chimerism rate of the stomach was 0.0618‰, which was extremely low. If the *Ghrelin* expression level were low, this would be difficult to detect by RT-PCR. Another possibility is that duck blastoderm cells could not differentiate into cell types that express the *Ghrelin* gene. The third possible

Fig. 6. RT-PCR analysis of various chimeric tissues and organs using duck tissue-specific *SPAG6* and *Ghrelin* primers. (A,B) RT-PCR analysis of duck blastodermal cells, turtle blastodermal cells and various duck and turtle tissues and organs using species-specific *GAPDH* primers and the duck tissue-specific *SPAG6* and *Ghrelin* primers, respectively. (A) M: Marker; Lanes 1–11: duck blastodermal cells, female duck gonad, male duck gonad, duck brain, small intestine, large intestine, kidney, stomach, liver, heart and muscle; Lanes 12–22: turtle blastodermal cells, female turtle gonad, male turtle gonad, turtle brain, small intestine, large intestine, kidney, stomach, liver, heart and muscle; Lane 23: blank control. (B) The lane assignments for the *SPAG6* gene analysis are the same as in A. For the *Ghrelin* gene, Lane 1: duck blastodermal cells; Lanes 2–4: duck stomach, small intestine and brain; Lanes 5–6: female duck gonad and male duck gonad; Lanes 7–11: duck large intestine, kidney, liver, heart and muscle; Lane 12: turtle blastodermal cells; Lanes 13–15: turtle stomach, small intestine and brain; Lanes 16,17: female turtle gonad and male turtle gonad; Lanes 18–22: turtle large intestine, kidney, liver, heart and muscle; Lane 23: blank control. (C) RT-PCR analysis of various tissues and organs of hatched duck–turtle chimeras. M: Marker; Lanes 1–7: gonad, large intestine, heart, muscle, pancreas, kidney and brain of a 1-yr-old female turtle; Lanes 8–17: small intestine, heart, muscle, pancreas, liver, kidney, stomach, large intestine, gonad and brain of a 1-yr-old male turtle; Lane 18: blank control.

explanation is that the transcriptional regulation of the duck *Ghrelin* gene may be disturbed in the chimeras. More research is required to test these ideas.

To our knowledge, this is the first study to report the production of Peking duck–Chinese soft-shelled turtle chimeras by the transfer of blastoderm cells. Production of interspecies chimeras is likely to contribute to our understanding of the evolutionary relationship between reptiles and birds and will be helpful for examining the early development of the turtle.

LIST OF ABBREVIATIONS

CYTB	cytochrome *b* gene
DMEM	Dulbecco's modified Eagle's medium
MEF2a	myogenic enhancer factor 2a gene
MEF2c	myogenic enhancer factor 2c gene
PBS	phosphate buffered saline
PGC	primordial germ cell
qPCR	quantitative real-time PCR
RT-PCR	reverse transcription PCR
SPAG6	sperm associated antigen 6 gene

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AUTHOR CONTRIBUTIONS

W.Z. and Z.L. conceived and designed the experiments. W.Z. and Z.L. wrote the paper and L.R. assisted with the writing. W.Z., J.Z. and L.R. performed the experiments and X.Y., Z.W., L.Y., F.Y., C.Q. and Q.S. assisted with the experiments. Z.Z. contributed materials.

COMPETING INTERESTS

No competing interests declared.

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