Improved method for ex ovo-cultivation of developing chicken embryos

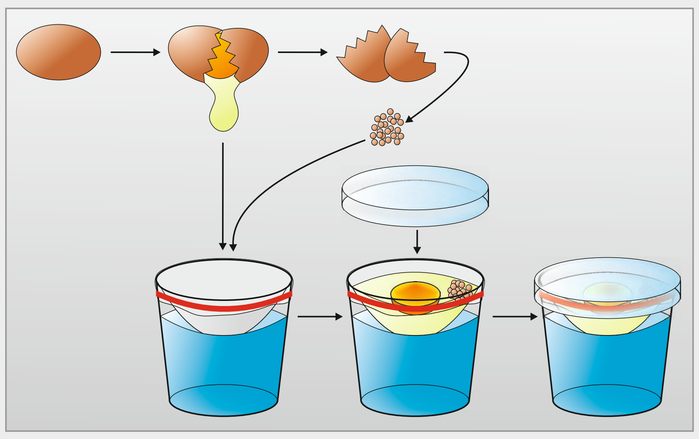
Timo Schomann[1], Firas Qunneis[2], Darius Widera[1], Barbara Kaltschmidt[2] and Christian Kaltschmidt[1]

1: Cell Biology, University of Bielefeld, Universitätsstr. 25, D-33501 Bielefeld, Germany
2: Molecular Neurobiology, University of Bielefeld, Universitätsstr. 25, D-33501 Bielefeld, Germany

Abstract

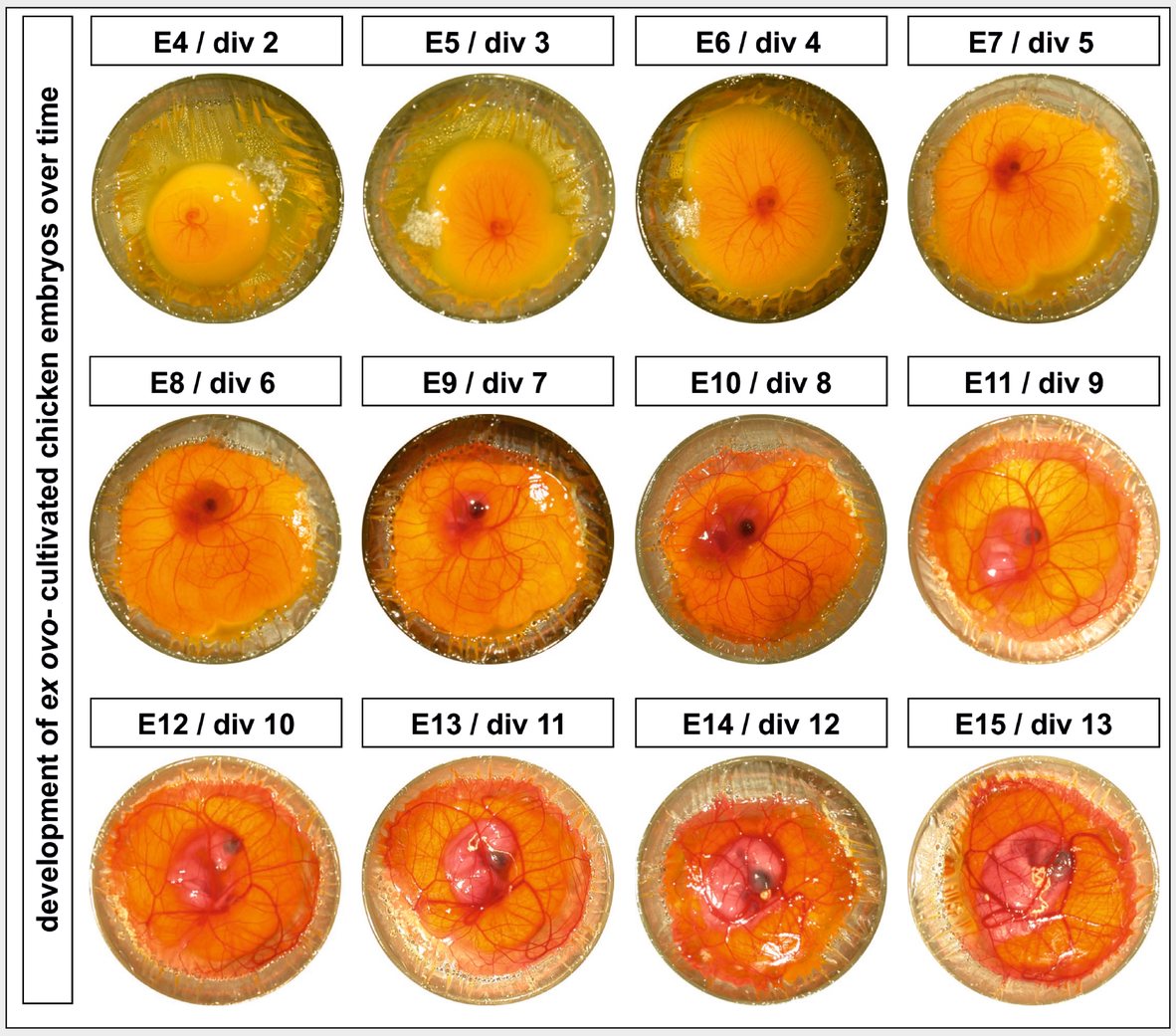
Prior to future use of human stem cells in regenerative medicine, investigations regarding their differentiation potential *in vivo* should be performed in animal models. Therefore, the inexpensive chicken embryo represents an ideal model organism. However, access to the chicken embryo is achieved by windowing the egg shell which results in limited visibility and accessibility in subsequent experiments. On the contrary, an *ex ovo*- culture system avoids such negative side effects.
Here we present an improved, *ex ovo*- cultivation method enabling the embryos to survive for at least 13 days *in vitro* (E15). Optimized cultivation of chicken embryos was achieved by preventing drop-related damage and leaking of egg contents and supplementation of nutrition using a defined amount of fine ground egg shell powder resulting in normal development of chicken embryos regarding their size and weight. We show that our *ex ovo*- approach closely resembles the development of the chicken embryo *in ovo*, as demonstrated by properly developed bones, cartilage, and nervous system at expected time- points.
Finally, we investigated the usability of our method for trans- species transplantation of neural crest-derived adult human inferior turbinate stem cells (ITSCs) by injecting these stem cells into late stages of *ex ovo*- incubated chicken embryos (HH26-HH28). Here, we demonstrated the integration of human cells for up to 4 days allowing experimentally easy investigation of the differentiation potential of such cells in the proper developmental context.
Taken together, we present an *ex ovo*- method that supports prolonged cultivation of properly developing chicken embryos, thus enabling observation of xenografted ITSCs in osteogenesis, chondrogenesis, and neurogenesis of the developing chicken embryo.

1. Schematic view of initial steps in *ex ovo*- cultivation of chicken embryos



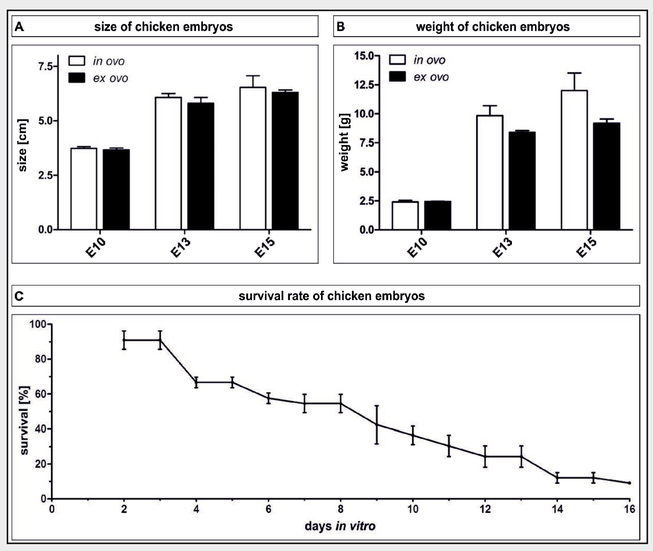
Prior to transfer chicken embryos were incubated at 37,8°C for 48 hours. Eggs were gently opened using a jigsaw. The indentation was expanded and the contents were carefully transferred onto the support film of the readily prepared *ex ovo*- containment. Ground egg shell of several eggs was added to the albumin and the experimental set- up was covered using a petri dish.

2. Morphologic development of *ex ovo*- cultivated chicken embryos



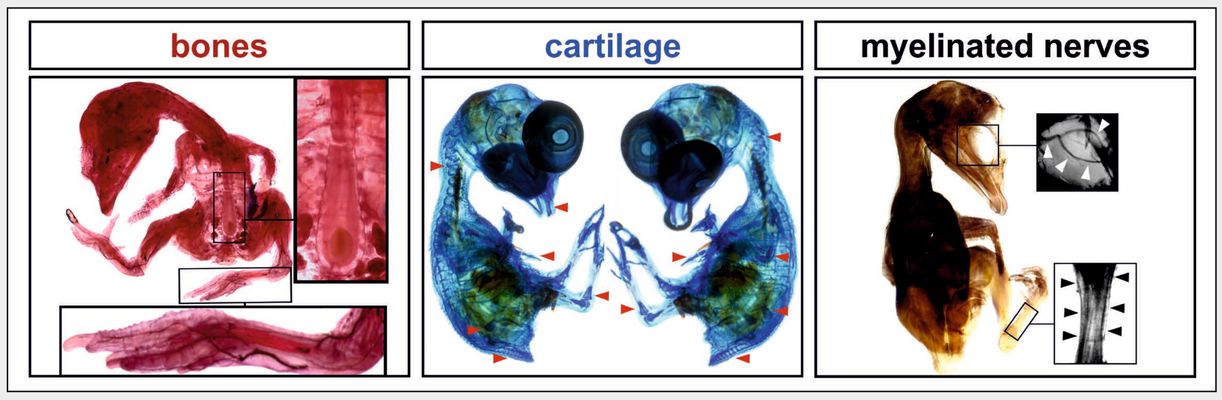
Starting with an easily visible chicken embryo at 2 days *in vitro* (div), correlating with embryonic day (E) 4, morphological changes in the development of *ex ovo*- cultivated embryos were distinguishable. After start of the *ex ovo*- cultivation the embryo developed normally for at least E15 / div 13 up to HH stage 41. The set of photos consists of a number of 4 different chicken embryos.

3. Statistical analysis of *ex ovo*- cultivated chicken embryos



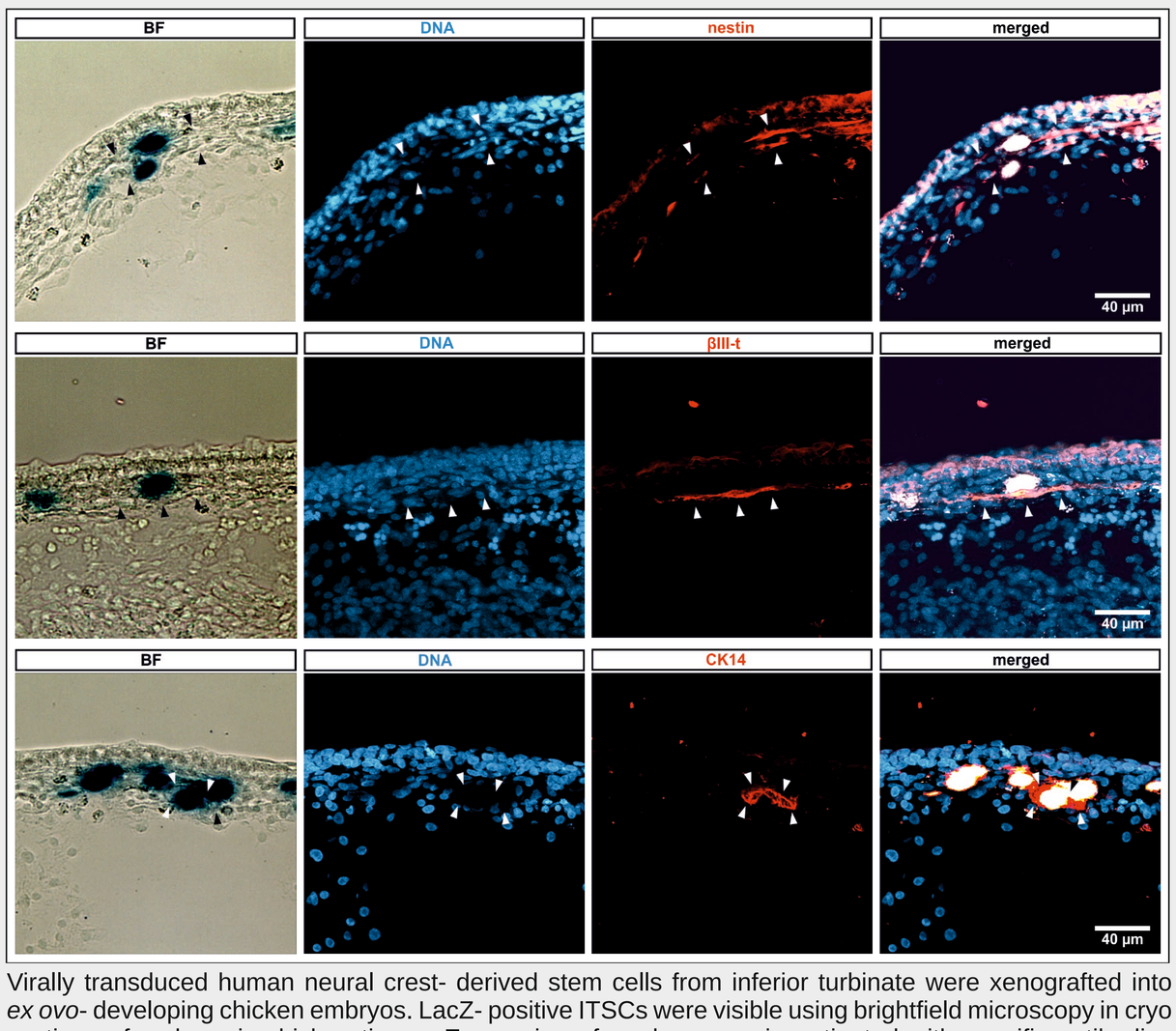
A: Size comparison of *in ovo*- and *ex ovo*- cultivated chicken embryos on days E10, E13, and E15. No significant differences in size were detectable between *ex ovo* and traditionally cultivated embryos. Error bar indicates SEM, n = 3. **B:** Chicken embryos cultivated *in ovo* and *ex ovo* were investigated regarding their size on days E10, E13, and E15 of incubation. *ex ovo*- cultivated embryos revealed no significant differences in weight compared to *in ovo*- cultivated chicken embryos. Error bar indicates SEM, n = 3. **C:** Survival rate of *ex ovo*- cultivated chicken embryos over time. After 48 h of traditional incubation egg contents were transferred into a *ex ovo*- containment and incubated for at least 13 days in vitro. Unfertilized eggs contents and contents without visible development of chicken embryos were discarded at least at div2 / E4. Error bar indicates SEM, n = 3, for E16 n = 1.

4. Organogenesis of bone, cartilage, and myelinated nerves



Chicken embryos were skinned and afterwards stained for bones using Alizarin Red S, cartilage using Alcian blue, and myelinated nerves using Sudan Black B. Alizarin Red S staining revealed ossification of the bones. In addition, Alcian blue demonstrates chondrification of cartilage and future bones (Arrowheads indicate cartilage stained by Alcian blue). Sudan Black B, a staining for myelinated nerves, was applied to investigate neurogenesis in *ex ovo*-cultivated chicken embryos. The optic nerve as well as nerves in the leg of the chicken embryos were positive for myelination, suggesting proper development.

5. Immunhistochemical analysis of xenografted ITSCs



Virally transduced human neural crest- derived stem cells from inferior turbinate were xenografted into *ex ovo*- developing chicken embryos. LacZ- positive ITSCs were visible using brightfield microscopy in cryo sections of embryonic chicken tissue. Expression of markers was investigated with specific antibodies against nestin, III-tubulin (III-t), and cytokeratin 14 (CK14).
Tissue- resident lacZ- positive ITSCs injected into developing chicken embryos were positive for the neural crest stem cell- marker nestin. In addition, xenografted ITSCs were positive for the ectodermal marker III-t. Integration of ITSCs in the basal layer of the dermis was underlined by expression of basal cell- marker CK14 in xenografted cells.

6. Summary

This novel *ex ovo*- cultivation approach enables long-term cultivation and investigation of chicken embryos. Chicken embryos cultivated *ex ovo* show normal growth regarding their weight and size. Furthermore, normal organogenesis of bones, cartilage, and myelinated nerves is underlined by specific stainings for the respective tissues.
Xenografted adult human nasal neural crest- derived ITSCs integrate and differentiate in late stages of *ex ovo*- developing chicken embryos.
This approach can be used for studies of development and stem cell research regarding integration into developing bones, cartilage formation, and development of nervous system.

7. References

Hauser, S., D. Widera, et al. (2012). "Isolation of novel multipotent neural crest-derived stem cells from adult human inferior turbinate." Stem Cells Dev 21(5): 742-756.

Nishikawa, K. C. (1987). "Staining Amphibian Peripheral-Nerves with Sudan Black-B - Progressive Vs Regressive Methods." Copeia 1987(2): 489-491.

Yalcin, H. C., A. Shekhar, et al. (2010). "An ex-ovo chicken embryo culture system suitable for imaging and microsurgery applications." J Vis Exp(44).