Use of marine cell lines as a source of factors that maintain pluripotency of zebrafish (*Danio rerio*) ES cell cultures

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Despite the many advantages of the zebrafish for studies of embryo development, toxicology and human disease, one deficiency of this model system is the absence of methods for introducing targeted mutations to create knockout lines of fish (Detrich *et al.*, Meth. Cell Biol. 59:3-10,1999). Development of gene targeting methods will complement other genetic approaches available with zebrafish to enhance the utility of this model system for studies of gene function. To develop a gene targeting strategy, we have established methods for the culture of zebrafish ES cells that remain pluripotent and germ-line competent *in vitro* (Ma *et al.*, PNAS 98:2461-2466, 2001; Fan *et al.*, Crit. Rev. Euk. Gene Exp. In press). Zebrafish germ-line chimeras have been generated from ES cells maintained for multiple passages in culture. A key component of this cell culture system is the use of feeder layers derived from the rainbow trout spleen cell line, RTS34st (Ganassin and Bols, In Vitro Cell Dev. Biol. Anim. 35: 80-86, 1999). The trout spleen cells release factors into the medium that maintain pluripotency and germ-line competency of the zebrafish embryo cell cultures.

To optimize the zebrafish ES cell culture system, factors that promote germ-line competency are being investigated. One strategy is to identify feeder layers and purified cytokines that are more effective than the RTS34st cells at maintaining germ-line competency of the zebrafish ES cells. In addition to testing feeder layers derived from tissues of freshwater fish, cell cultures initiated from marine species are being examined as a source of factors that inhibit differentiation and promote germ-line competency of the zebrafish cells. The objective of this research is to determine if cell cultures derived from dogfish shark (*Squalus acanthius*) and skate (*Raja erinacea*) tissues produce factors that influence the differentiation of zebrafish ES cell lines. Conditioned medium obtained from the shark or skate cell cultures (obtained from Dr. David Barnes, MDIBL) was added to zebrafish ES cells maintained in the absence of RTS34st feeder layers and the ES cells were assayed by RT-PCR for expression of pou2, a marker of pluripotency. Conditioned medium collected from primary cultures of dogfish shark and skate liver, spleen, brain, gut and embryos was added to the zebrafish ES cell cultures. The initial results indicate that medium conditioned by shark embryo cells was able to maintain pou2 expression in the ES cells for at least 5 days in the absence of RTS34st feeder layers. After 5 days, ES cells maintained in the presence of 50% shark embryo cell conditioned medium without a feeder layer expressed pou2 at a level that was similar to control ES cells grown on the RTS34st feeders. Conditioned medium collected from the other tissues was not able to promote pou2 expression. To confirm these results, ES cells cultured in the presence of shark embryo cell conditioned medium are being evaluated for the capacity to generate germ-line chimeras following injection into host embryos.

Identification of a cell culture producing factors able to preserve germ-line competency and pluripotency of the zebrafish ES cells will contribute to the development of a gene targeting system and enhance the utility of the zebrafish model for studies of gene function.

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