Receptor kinase expression in germ-line competent zebrafish (*D. rerio*) ES cells

J. Denry Sato[1], M. Christine Chapline[1], Peter Alestrom[2], Lianchun Fan[3], and Paul Collodi[3]

[1]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672
[2]Dept. of Biochemistry, Norwegian School of Veterinary Science, N-0033 Oslo, Norway
[3]Dept. of Animal Sciences, Purdue University, West Lafayette IN 47907

We are identifying extracellular and genetic factors that confer germ-line competency to pluripotent zebrafish (*Danio rerio*) embryonic stem (ES) cells in a project to optimize the existing cell culture system for zebrafish ES cells developed for use in the production of gene-targeted knockouts[1,2]. One of the approaches we are using to achieve this objective is to generate a profile of the growth factor receptors that are expressed in germ-line competent zebrafish ES cells. This information will tell us to which growth factors these cells can potentially respond, and it will suggest receptor kinase-activated signaling pathways that may be involved in the maintenance of germ-line competency. The factors identified in this work will be tested in the culture system for the ability to enhance the production of zebrafish germ-line chimeras. Since zebrafish ES cell cultures are the only non-murine cells available that successfully contribute to the germ cell lineage of a host embryo, they provide a unique system to study factors controlling germ-line competency. In general, gene-targeting techniques are not available in non-murine species due to the absence of germ-line competent ES cell lines. Analysis of the factors conferring germ-line competency to zebrafish ES cell cultures will provide information that can be applied to the development of ES cell cultures and gene-targeting technology in other animal models.

Protein kinases in eukaryotic cells are divided into serine/threonine-specific and tyrosine-specific classes depending on their substrate specificities[3,4]. These kinases have a high degree of similarity in their kinase or catalytic domains, which consist of 250-300 amino acids. Since the discovery of the EGF receptor tyrosine kinase by Stanley Cohen and colleagues[5,6], numerous receptors for growth factors and hormones have been found to transmit intracellular signals via intrinsic or non-covalently associated tyrosine kinases. These signaling cascades ultimately change gene expression patterns in cells by activating transcription factors. Amongst the approximately 32,000 protein coding genes in the human genome, there are 520 protein kinase genes and more than 90 protein tyrosine kinase genes of which 58 encode cell surface receptor molecules[7]. The serine/threonine-specific receptor protein kinases that have been identified thus far all bind members of the TGF-beta superfamily of ligands[8]. The hypothesis that cell surface receptors with intrinsic kinase activities and their ligands are intimately involved in cell proliferation and differentiation in vertebrate development has received much experimental support over that past 25 years[9-12]. For example, members of the bone morphogenetic protein (BMP) subgroup of the TGF-beta superfamily[10] and the FGF family of growth factors[11] have been found to be involved in mesoderm induction during gastrulation and in limb development and organogenesis later in development. Recently, Ying, et al.[13] have found BMPs contribute the maintenance of murine ES cell pluripotency by inducing the expression of inhibitor of differentiation (Id) proteins.

We are using RT-PCR with degenerate oligonucleotide primers corresponding to conserved receptor tyrosine kinase sub-domains[14] to clone fragments of specific receptor cDNAs from

RNAextracted from germ-line competent zebrafish ES cells maintained on trout spleen feeder cells[1]. The ES cells were harvested with EDTA solution, which caused the ES cells to dissociate from the underlying adherent trout feeder cells. This method yielded ES cell preparations of 90% or greater purity. Single-stranded oligo(dT)-primed cDNA was amplified with degenerate PCR primer pools corresponding to sub-domain VIB (384x17mer) and VIII (288x17mer) sequences taken from all annotated zebrafish receptor tyrosine kinase cDNAs in the GenBank database. Aliquots of the PCR product pool were subjected to an additional 30 cycles of amplification with specific primers from sub-domain VIB and VIII sequences in the epidermal growth factor receptor (EGFR), fibroblast growth factor receptor-1 (FGFR1) and platelet-derived growth factor receptor (PDGFR) alpha. The PCR products were resolved by electrophoresis in a 1% agarose gel (Fig. 1A). A unique ethidium bromide-stained band of about 160bp was detected in each specific PCR reaction (lanes 1-3) but not in the control reaction, which contained no template DNA (lane 4). The stained band in the control reaction was most likely a primer dimer product.

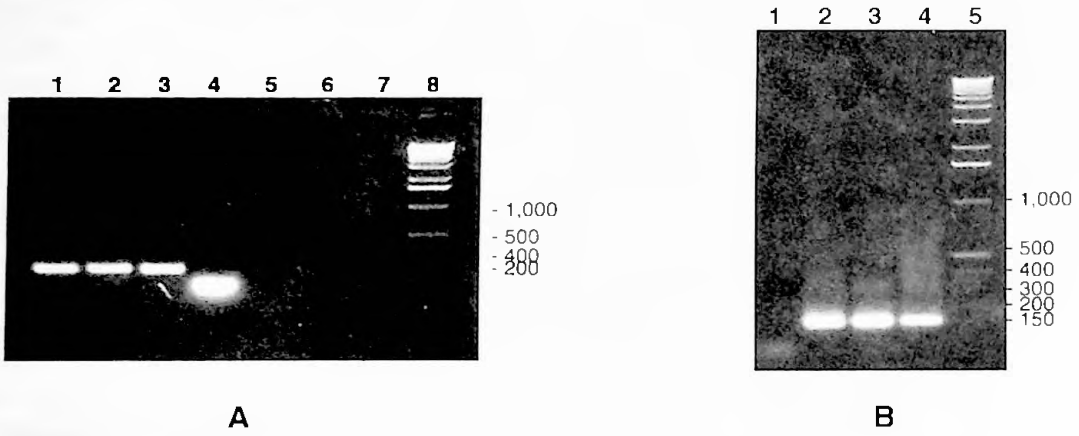

Figure 1. PCR products generated with specific primers to zebrafish receptor tyrosine (A) and serine/threonine (B) kinases from ES cell cDNA amplified with degenerate primer pools. (A) 1, EGFR primers; 2, FGFR1 primers; 3, PDGFR alpha primers; 4, control with no DNA template; 8, DNA ladder. (B) 1, control with no DNA template; 2, ActRIIB primers; 3, BMPR1a primers; 4, TGFbRII primers; 5, DNA ladder. The sizes of DNA markers are provided in base pairs.

A similar agarose gel of PCR products generated with specific primers corresponding to sub-domains VIB and VIII of the receptor serine/threonine kinases for activin (ActRIIB, lane 2), bone morphogenetic proteins (BMPR1a, lane 3), and transforming growth factor-beta (TGFbRII, lane 4) is shown in Fig. 1B. Again a unique ethidium bromide-stained band of approximately 160bp was detected in each specific PCR reaction (lanes 2-4) but not in the control reaction, which contained no template DNA (lane 1). The templates for these PCR reactions were cDNA fragments amplified with degenerate PCR primer pools corresponding to sub-domain VIB (48x17mer) and VIII (108x19mer) from all annotated zebrafish receptor serine/threonine kinase cDNAs in GenBank.

In another experiment, specific receptor serine/threonine kinase fragments were cloned directly from PCR products amplified with degenerate primers corresponding to sub-domains VIB and VIII, and they were identified by DNA sequencing. Of ten clones sequenced, three corresponded to bone morphogenetic protein receptor 1a (BMPR1a) (mean sequence identity=98%), three corresponded to BMPR1b (mean sequence identity=97%), and three corresponded to activin receptor type II-like protein (mean sequence identity=100%). These levels of sequence identity indicate that the degenerate

primers amplified *bona fide* zebrafish receptor kinase templates. The last clone exhibited a lower degree of similarity (81% identity) to zebrafish BMPR1a, which indicated that it represented either a related zebrafish serine/threonine kinase or BMPR1a expressed by the trout feeder cells. This question can be resolved by sequencing trout PCR products amplified with specific zebrafish primers. If zebrafish receptor kinase target sequences are identical to their counterparts in rainbow trout, then mRNA from a pure sorted population of zebrafish ES cells will be needed as a template for cDNA synthesis. Our results indicate that degenerate PCR primer pools corresponding to conserved regions of receptor kinases can be used successfully to clone and identify cDNA fragments for closely-related (eg., BMPR1a and BMPR1b) and distantly-related (eg., EGFR and PDGFR alpha) receptor molecules. Ligands for the identified receptor kinases will be tested *in vitro* for the ability to enhance the production of zebrafish germ-line chimeras.

We thank Christine Smith for sequencing RT-PCR products in the Marine DNA Sequencing Center. JDS is supported by grant P20-RR16463 from the National Center for Research Resources, and he is an investigator of the Center for Membrane Toxicity Studies (NIEHS grant P30-ES03828). PC is supported by grant RO1-GM69384 from the Institute of General Medicine, and he is the recipient of an MDIBL New Investigator Award.

1. Ma, C., Fan, L., Ganassin, R., Bols, N. and Collodi, P. Production of zebrafish germ-line chimeras from embryo cell cultures, *Proc. Natl. Acad. Sci. USA* 98, 2461-2466 (2001).
2. Fan, L. and Collodi, P. Progress towards cell-mediated gene transfer in zebrafish, *Brief Functional Genom. Proteom.* 1, 131-138 (2002).
3. Hanks, S.K., Quinn, A.M., and Hunter, T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 241, 42-52 (1988).
4. Hanks, S.K., and Hunter, T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 9, 576-596 (1995).
5. Carpenter, G., King, L. Jr, and Cohen, S. Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. *Nature* 276, 409-410 (1978).
6. Ushiro, H., and Cohen, S. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J. Biol. Chem.* 255, 8363-8365 (1980).
7. Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. The protein kinase complement of the human genome. *Science* 298, 1912-1934 (2002).
8. Massague, J., and Weis-Garcia, F. Serine/threonine kinase receptors: mediators of transforming growth factor beta family signals. *Cancer Surv.* 27, 41-64 (1996).
9. Hogan, B.L., Blessing, M., Winnier, G.E., Suzuki, N., and Jones C.M. Growth factors in development: the role of TGF-beta related polypeptide signaling molecules in embryogenesis. *Dev. Suppl.*, 53-60 (1994).
10. Hogan, B.L. Bone morphogenetic proteins in development. *Curr. Opin. Genet. Dev.* 6, 432-438 (1996).
11. Martin, G.R. The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* 12, 1571-1586 (1998).
12. Blume-Jensen, P., and Hunter, T. Oncogenic kinase signaling. *Nature* 411, 355-365 (2001).
13. Ying, Q.L., Nichols, J., Chambers, I., and Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115, 281-292 (2003).
14. Hardie, G., and Hanks, S. *The Protein Kinase FactsBook*, Academic Press, San Diego, CA (1995).