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Surgical manipulation of mammalian embryos *in vitro*

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ABSTRACT Whole-embryo culture systems are useful in the fields of not only embryology but also teratology, toxicology, pharmacology, and physiology. Of the many advantages of whole-embryo culture, we focus here on the surgical manipulation of mammalian embryos. Whole-embryo culture allows us to manipulate mammalian embryos, similarly to fish, amphibian and avian embryos. Many surgical experiments have been performed in mammalian embryos *in vitro*. Such surgical manipulation alters the destiny of morphogenesis of the embryos and can answer many questions concerning developmental issues. As an example of surgical manipulation using whole-embryo culture systems, one of our experiments is described. Microsurgical electrocauterization of the deep preaxial mesodermal programmed cell death zone (fpp) in the footplate prevented the manifestation of polydactyly in genetic polydactyly mouse embryos *(Pdn/Pdn)*, in which fpp was abolished.

KEY WORDS: *whole-embryo culture, embryo surgery, polydactyly*

Introduction

The whole mammalian embryo culture system developed mainly by New and colleagues (New and Coppola, 1970; Cockroft, 1973, 1976, 1979, 1988; New *et al.*, 1973, 1976a,b; Steele and New, 1975; New and Cockroft, 1979) has the following advantages over *in vivo* experiments: 1) continuous observation of development, 2) surgical manipulation of the embryos, 3) omission of maternal factors, 4) exposure to agents at the same developmental stage, 5) direct exposure of the embryos to agents, bypassing maternal metabolism, and 6) direct injection of agents into precise sites within the embryos.

In this review, we focus on surgical manipulation; whole-embryo culture systems have allowed surgical methods to be used with mammalian embryos to investigate normal and abnormal development.

Deuchar analyzed the mechanism of axial rotation by embryo surgery in rats *in vitro* (Deuchar, 1971, 1975). In rat embryos on days 9.5-10.5 of gestation, axial rotation was greatly inhibited when the cervical level was incised transversely. This suggested that the cervical region possesses the contractile motive force that initiates axial rotation in the rat embryo.

Deuchar removed rat anterior limb buds through a slit in the yolk sac membrane *in vitro*, and almost complete regeneration of the removed limb buds was observed (Deuchar, 1976). This experiment showed that the early rat embryo possesses limb bud regenerative capacity. Later, Wanek *et al.* (1989) showed partial regeneration of the phalanges after amputation through the prospective phalangeal tissue in the mouse footplate on day 12 of gestation using *exo utero* surgical methods.

Moore and Metcalf (1970) cultured mouse embryos explanted on day 7.5 of gestation with or without the yolk sac, or yolk sac alone, and demonstrated that hemopoietic stem cells were generated in the yolk sac and migrated into the embryo.

Whole-embryo culture systems have also allowed the marking of embryonic cells with radioisotopes or various dyes *in vitro*, and has thus facilitated tracing of the destinations and migration routes of undifferentiated cells (Beddington, 1981,1994; Lawson *et al.*, 1986, 1987; Tan and Morriss-Kay, 1986; Osumi-Yamashita *et al.*, 1994).

Eto *et al.* (1981) implanted an eyelash soaked in tunicamycin solution into the nasal placode of the cultured rat embryo, and induced unilateral cleft lip. Recently, Augustine *et al.* (1993, 1995) and Sadler *et al.* (1995) injected antisense oligo DNA of *Wnt-1, Wnt-3a, En-1, and En-2* into the amniotic cavity of mouse embryos *in vitro*, and inactivated the function of *Wnt* and *En* genes inducing abnormal development in the craniofacial structures, central nervous system and heart, and shortening of the embryonic axis. Thus, whole-embryo culture systems allow application of test agents into precise sites within the embryos.

Prevention of genetic polydactyly by surgical treatment of mouse footplate during embryogenesis *in vitro*

Homozygous Polydactyly Nagoya mice *(Pdn/Pdn)* show preaxial polydactyly of the duplicated or triplicated metacarpal/metatarsal type in the fore- and hindlimbs, and heterozygotes (*Pdn*/+) show

Abbreviations used in this paper: Pdn, polydactyly Nagoya; fpp, foyer préaxial primaire: deep preaxial mesoderm; AER, apical ectodermal ridge.

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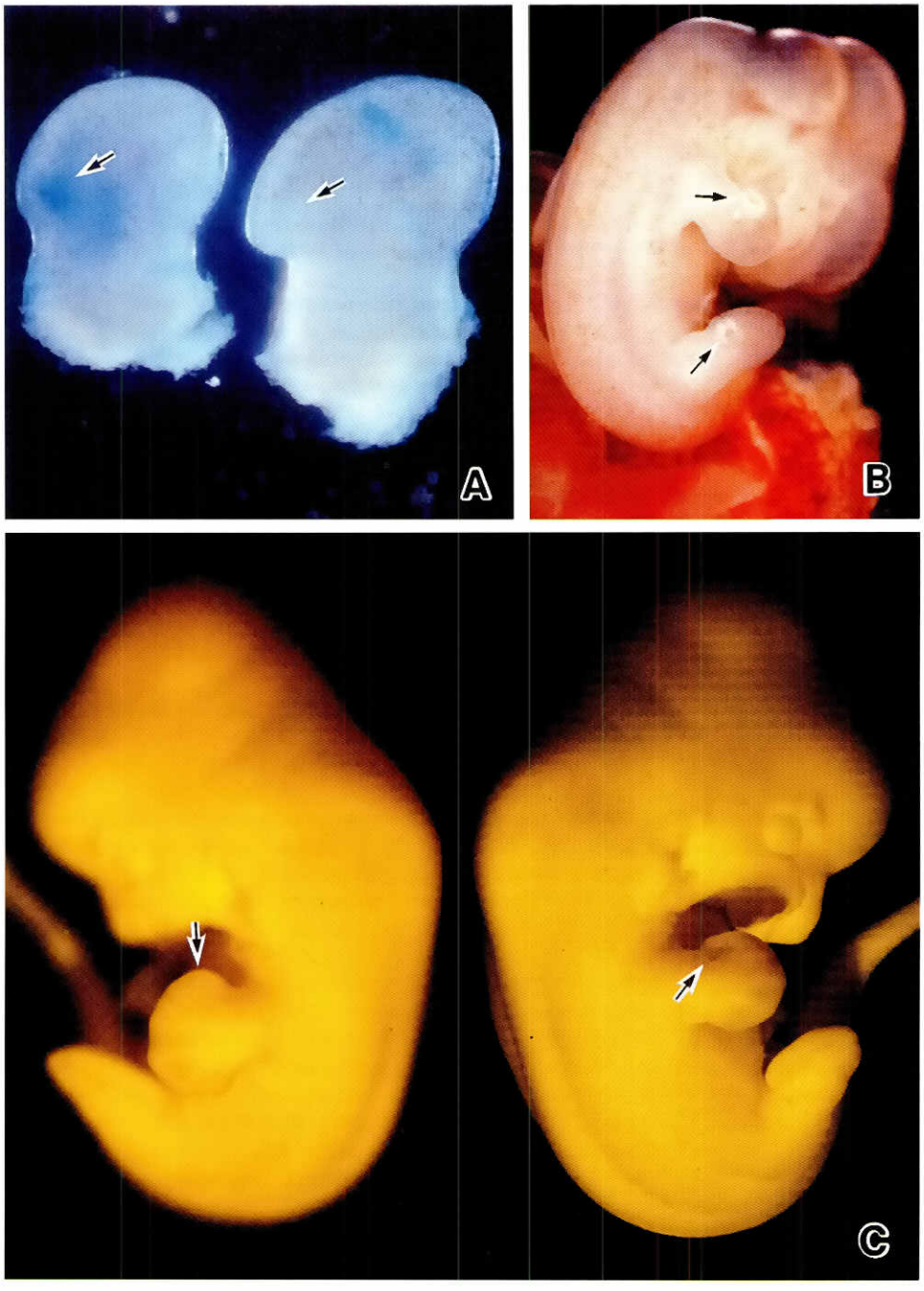

Fig. 1. Prevention of genetic polydactyly by electrocauterization in cultured mouse embryos. (A) *Normal (+/+) mouse hindlimb plate (left side) on day 12 of gestation showing deep preaxial mesodermal cell death zone (fpp, arrow). Pdn/Pdn hindlimb plate (right side) on day 12 of gestation showing no fpp (arrow). Nile blue vital-stained specimens.* **(B)** *A mouse embryo on day 11.5 of gestation just after electrocauterization. Injuries of the preaxial mesoderm in the right fore- and hindfoot plates are indicated by arrows.* **(C)** *Pdn/Pdn embryo cultured in the rotator culture system for 20 h from day 11.5 of gestation. Left non-treated forefoot plate (left side) showing a preaxial protrusion and/or extradigital ray (arrow). On the other side, the treated right forefoot plate exhibits 5 digital rays. The arrow indicates electrocauterization scar.*

one extra digit of the phalangeal type preaxially in the hindlimb, and deformity of the distal phalanx of the 1st digit in the forelimb. In the fore- and hindlimbs of *Pdn/Pdn* embryos on days 11-12 of gestation, the normal patterns of programmed cell death in the preaxial apical ectodermal ridge (AER) and deep preaxial mesoderm (foyer préaxial primaire, fpp) are disrupted, and involution of the preaxial AER is delayed (Naruse and Kameyama, 1982). The abolishment of the fpp is generally considered to lead to polydactyly (Milaire and Rooze, 1983). The evidence favoring this is derived from previous findings that regimes which induce preaxial polydactyly e.g. drugs (Scott *et al.*, 1977, 1980; Klein *et al.*, 1981; Scott, 1981; Wise and Scott, 1982) or genes (Rooze, 1977; Knudsen and Kochhar, 1981; Naruse and Kameyama, 1982), result in an absence of this zone of programmed cell death. Scott *et al.* (1980) reported that the ectodermal cells which did not die on schedule might provide an additional influence on the underlying mesoderm (fpp) sufficient to prevent these cells from dying. It was further supposed that because cell death was prevented, the proliferative cell population in preaxial mesoderm increased and there is a surplus of preaxial mesodermal cells resulting in excessive digits. This hypothesis conforms to the observed polydactyly manifested in *Pdn/Pdn* embryos. That is, normal patterns of programmed cell death in the preaxial AER and fpp were abolished in *Pdn/Pdn* embryos (Fig. 1A). Delayed involution of preaxial AER might cause the abolishment of fpp, which in turn could induce polydactyly in *Pdn/Pdn*.

To induce cell death in the fpp region artificially, destruction of the fpp region of the right fore- and hindfoot plates of *Pdn/Pdn*, *Pdn/+* and +/+ embryos was performed by electrocauterization on day 11.5 of gestation (Fig. 1B), and the embryos, exteriorized from the yolk sac through a slit (Cockroft, 1973), were cultured with 100% rat serum in the rotator culture system (New and Cockroft, 1979) for 20 h. The non-treated left footplates served as controls. In *Pdn/Pdn* embryos, the non-treated left footplates showed abnormal protuberance and/or extra digital rays preaxially (Fig. 1C, left) but the treated right footplates did not exhibit these abnormal characteristics (Fig. 1C, right). Artificial cell death in the fpp region prevented the manifestation of genetic polydactyly in *Pdn/Pdn* mouse embryos. Thus, the hypothesis that the abolishment of the fpp leads to polydactyly was directly verified using the whole-embryo culture system (Naruse and Kameyama, 1986).

Recently, molecular genetics in the pattern formation of digits has progressed strikingly in avian embryos, and this knowledge is spreading to mammalian embryos. It has been shown that the inducer from the AER is FGF (Niswander *et al.*, 1993; Fallon *et al.*, 1994), and that Sonic hedgehog *(Shh)* mediates ZPA (zone of polarizing activity) (Riddle *et al.*, 1993; Tabin, 1995). Sonic hedgehog protein may let the *Hox-5* genes be expressed to form the digits from posterior to anterior (Dollé *et al.*, 1989). It is apparent that *Shh* and *Fgf-4* maintain their expressions reciprocally, and these gene expressions coordinate growth of the limbs and pattern formation of the digits (Laufer *et al.*, 1994; Niswander *et al.*, 1994). Polydactyly mutant mice, Hemimelia extra toes *(Hx)* and Extra toes *(Xt)*, revealed ectopic expressions of *Shh* and *Fgf-4* genes, and an additional ZPA at the anterior margin of the limb buds (Masuya *et al.*, 1995). The gene responsible for *Pdn/Pdn* has been shown to be *Gli3* (Krüppel gene family, Naruse and Keino, 1995). Alexandre *et al.* (1996) reported that the best candidate for a transcription factor that mediates hedgehog signaling in *Drosophila* is the product of the cubitus interruptus *(ci)* gene, a zinc finger protein that exhibits significant homology to protein products of the vertebrate *Gli* gene family. Masuya *et al.* (1995) speculated that *Gli3* might suppress ZPA activity specifically at the anterior margin of the limb bud in normal mouse embryos. It is possible that interactions between *Gli3* and *Shh* genes may have a role in the abolishment of fpp, resulting in polydactyly.

Fetal surgery *exo utero*

Whole-embryo culture is limited with respect to culture period as described by New (1978), so it is important to start the culture just before the morphogenesis of the organs of interest. It is very difficult to culture fetuses in late gestation, and thus *exo utero* fetal surgery has been developed (Muneoka *et al.*, 1986, 1989; Naruse and Kameyama, 1989, 1990; Naruse and Tsutsui, 1989; Wanek *et al.*, 1989; Naruse and Keino, 1993, 1995; Naruse *et al.*, 1995). Fifty to seventy percent of fetuses treated surgically *exo utero* on day 13 of gestation develop until day 18 of gestation. To rear them further, fetuses can be recovered from the dam's abdominal cavity and incubated on a warm plate. It is subsequently possible to rear them further using a foster mother that has just given birth (Naruse and Tsutsui, 1989; Naruse *et al.*, 1995). It is very difficult to obtain development of mouse embryos which have received surgery *exo utero* before day 13 of gestation, so the success rate is very low (Naruse and Kameyama, 1989).

Summary

Of the many advantages of whole-embryo culture systems, we focus on embryo surgery in this review. Whole-embryo culture allows the surgical manipulation of mammalian embryos, and embryo surgery can alter the destiny of morphogenesis.

Homozygous Polydactyly Nagoya *(Pdn/Pdn)* mice show abnormal programmed cell death patterns in the limb plates, and abolishment of the preaxial apical ectodermal ridge and the deep preaxial mesodermal cell death zone. Electrocauterization in the deep preaxial mesoderm of the limb plates prevents the manifestation of polydactyly in *Pdn/Pdn* mice in whole-embryo culture.

Thus, embryo surgery *in vitro*, one of the many techniques made possible by whole-embryo culture systems, is a useful method with which to elucidate certain aspects of development.

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