Microarray Analysis of Murine Limb Bud Ectoderm and Mesoderm after Exposure to Cadmium or Acetazolamide

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BACKGROUND: A variety of drugs, environmental chemicals, and physical agents induce a common limb malformation in the offspring of pregnant mice exposed on day 9 of gestation. This malformation, postaxial, right-sided forelimb ectrodactyly, is thought to arise via an alteration of hedgehog signaling. **METHODS:** We have studied two of these teratogens, acetazolamide and cadmium, using the technique of microarray analysis of limb bud ectoderm and mesoderm to search for changes in gene expression that could indicate a common pathway to postaxial limb reduction. **RESULTS:** Results indicated a generalized up-regulation of gene expression after exposure to acetazolamide but a generalized down-regulation due to cadmium exposure. An intriguing observation was a cadmium-induced reduction of Mt1 and Mt2 expression in the limb bud mesoderm indicating a lowering of embryonic zinc. **CONCLUSIONS:** We propose that these two teratogens and others (valproic acid and ethanol) lower sonic hedgehog signaling by perturbation of zinc function in the sonic hedgehog protein. *Birth Defects Research (Part A) 85:588–598, 2009.* © 2009 Wiley-Liss, Inc.

Key words: microarray; cadmium; acetazolamide; limb buds

INTRODUCTION

A number of chemical and physical agents, many of them documented human teratogens, induce a common limb phenotype, postaxial forelimb ectrodactyly, in rodent fetuses exposed during midgestation (Bell et al., 1997). The syndrome is manifest as loss of digits beginning posteriorly (digit 5) and progressing anteriorly according to dose, primarily in the forelimb, with a definite asymmetrical predominance on the right side. At high dosage there is often loss of the postaxial bone of the zeugopod (forearm), the ulna. It is our contention that this limb phenotype is caused by a down-regulation of hedgehog (Hh) signaling based on (1) restricted sonic hedgehog (*Shh*) expression to the posterior mesenchyme of the vertebrate limb bud (Echelard et al., 1993; Riddle et al., 1993); (2) recognition that Shh signaling is equivalent to polarizing activity (Riddle et al., 1993), the force that directs anterior/posterior patterning of the limb (Saunders and Gasseling, 1968; Tickle et al., 1975); (3) depression of *Shh* expression in the limb bud of *Wnt7a* null embryos destined to exhibit postaxial forelimb ectrodactyly with right-sided predominance (Parr and McMahon, 1995); (4) resemblance of the postaxial forelimb ectrodactyly phenotype to the limb phenotype in *Shh* null fetuses (Chiang et al., 2001; Kraus et al., 2001); (5) induction of postaxial forelimb ectrodactyly in the murine fetus exposed to jervine (ten Berge et al., 2001), an inhibitor of Hh signaling (Cooper et al., 1998); (6) downregulation of *Shh* expression in ethanol-induced postaxial forelimb ectrodactyly (Chrisman et al., 2004); (7) loss of polarizing activity from the limb bud of murine embryos exposed to acetazolamide (Bell et al., 1999) or cadmium (Scott et al., 2005); and (8) down-regulation of Hh signaling, as measured by a Gli-Luc reporter, in the limb bud of murine embryos exposed to acetazolamide (Bell et al., 2005) or cadmium (Scott et al., 2005).

Hedgehog signaling is a crucial component for proper development of many, perhaps most, developing organs (McMahon et al., 2003); and perturbation of Hh signaling leads to congenital malformations in the clinic (Cohen, 2003; McMahon et al., 2003; Nieuwenhuis and Hui, 2004).

Hedgehog signaling, from the precisely restricted expression pattern of the mammalian family homologs, sonic, Indian, and desert hedgehogs, to the elaborate regulation of Hh signaling effectors, the Gli family genes, is

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a very complex cell biologic phenomenon that includes auto-processing, lipid modification, multimerization, secretion, extra-cellular matrix interactions, gradient formation, receptor binding and internalization, phosphorylation, intracellular transport, proteolytic cleavage, and proteosomal degradation (Ingham and McMahon, 2001; Cohen, 2003; Hooper and Scott, 2005). This complexity provides numerous potential sites where toxicity from diverse chemical or physical agents could act to derail Hh signaling. Conversely the agents that induce postaxial forelimb ectrodactyly could induce a common toxicological response that would perturb some component of this complex pathway and lead to down-regulation of Hh signaling. We have examined this latter possibility, proposing that these agents, at high dosage, induce a stress response in limb bud cells, which acts by an obscure mechanism to down-regulate Hh signaling. The concept of a stress response as the common denominator initiating lowered Shh signaling leading to postaxial forelimb ectrodactyly was suggested by the known ability of some ectrodactyly-inducing agents to initiate a stress response in many systems including hyperthermia (Sonna et al., 2002), cadmium (Beyersmann and Hechtenberg, 1997), and ethanol (Calabrese et al., 1996).

We selected microarray analysis as a tool to search for changes in gene expression that might underlie the induction of postaxial forelimb ectrodactyly. We used the Affymetrix oligonucleotide array system and examined gene expression patterns in the two tissue components that contribute to the vertebrate limb bud, covering ectoderm, and underlying mesoderm. There is a long history of interactions between these tissues that are required for normal limb morphogenesis (Zwilling and Hansborough, 1956), and it remains unclear which tissue might be the initial site of teratogen attack. We examined gene expression in these two tissues 3 and 12 hr after teratogen administration. These intervals were chosen to elucidate early changes in gene expression after cellular injury (3 hr), and to uncover genes or signaling pathways that are slower to respond or represent a second tier response (12 hr).

To identify gene expression changes that might be integral to teratogen-induced postaxial forelimb ectrodactyly, we studied acetazolamide and cadmium. These agents were chosen primarily because they induce a high frequency of this common phenotype, that is, >75% of the fetuses at E18.5 will have missing posterior digits on the right forelimb.

METHODS

Animals

Studies were conducted in C57BL/6NCrIBR mice (Charles Rivers, Wilmington, MA). Individual males were placed with three females for the last 2–3 hr of the 12 hr dark cycle (10 PM to 10 AM). The presence of a vaginal plug indicated a successful mating, and 9 AM was considered time 0 of pregnancy.

Teratology Study

At E9.5, pregnant mice were given a single intraperitoneal injection of acetazolamide suspended in vegetable oil, 400 mg/kg (Sigma, St. Louis, MO). At E18 the dams were sacrificed by isofluorane overdose. The fetuses were removed from the uterus and prepared for skeletal analysis by double staining of cartilage and bone (Kuczuk and Scott, 1984). A teratology study for cadmium sulfate in the same strain of mouse was reported in Scott et al. (2005).

Sample Collection and Preparation

At E9.5 pregnant mice were given a single intraperitoneal injection of $CdSO_4$ (Sigma) in phosphate buffered saline (PBS; 4 mg/kg) or acetazolamide (400 mg/kg). Control mice received vehicle only (10 ml/kg). Three or 12 hours later, the mice were euthanized by an overdose of isofluorane.

Embryos were removed and dissected free from their extraembryonic membranes in PBS, somites counted, and right forelimbs staged according to Wanek et al. (1989). Only stage 1.5–1.75 right forelimbs from 25–28 somite embryos were collected at 3 hr posttreatment, and only stage 2.5–2.75 right forelimbs from 29–31 somite embryos were collected at 12 hr posttreatment. The staging was corroborated by two investigators (C.S. and W.S.).

The right forelimb of the appropriate stage was excised from the body wall. To separate ectodermal hulls from the mesoderm, limbs were trypsinized at 4°C (2% crude trypsin in Mg^{++}/Ca^{++}-free Tyrode's buffer; 30 minutes for stage 1.5 limbs and 45 minutes for stage 2.5 limbs), followed by a 20-minute incubation at 4°C in 5% fetal bovine serum in Tyrode's buffer containing Mg^{++} and Ca^{++}. Ectoderm was removed from the mesoderm using watchmaker forceps. Tissues were rinsed in PBS at 4°C, pooled, and frozen at −70° C. Total RNA was isolated using the Stratagene nanoprep RNA isolation kit and quantitated using Ribogreen (Invitrogen, Grand Island, NY). Sample sizes contained 25–30 ectodermal hulls or mesodermal cores.

Microarray

Samples were given to the Cincinnati Children's MicroArray Core for amplification and hybridization. Integrity of the RNA samples was analyzed by electrophoresis on an Agilent 2100 Bioanalyzer before microarray analysis. The Amersham Codelink kit was used to amplify and label 50-ng samples. Because of the small sample size, ectoderm samples were amplified twice. Labeled product was hybridized to the Affymetrix MG-U74Av2 high-density Oligonucleotide array (12,488 probe sets). Three independent biologic samples were prepared for each treatment, time, and tissue. For the third biologic sample for each treatment, the hybridization buffer had been changed by Affymetrix to improve the quality of the arrays. Therefore, the first two samples of each treatment (Trial 1) and the last sample of each treatment (Trial 2) were normalized independently and then analyzed.

Raw CEL files were analyzed using the Robust Multi-Array Analysis (RMA) algorithm, developed and described by Irizarry et al. (2003). Relative gene expression was determined by using the RMA protocol implemented in RMAExpress (written by Ben Bolstad from the University of California at Berkeley) with default background subtraction and quantile normalization. The signal intensity of each of the probe sets was first transformed from a log base 2 to linear values and then further normalized to the mean of the intensities determined for that gene across the corresponding PBS or oil control samples in two separate trials respectively.

The expression data was analyzed using GENESPRING ver. 7.2 (Silicon Genetics, Redwood City, CA). Signifi-

cantly altered probe sets for the teratogen-exposed samples were obtained using the following criteria; (1) At least four of the six samples in any comparison were considered present; (2) fold change is equal to or >2; and (3) signal should be above (teratogen-induced increase) or below (teratogen-induced decrease) signal control by at least two standard deviations of the control mean or by $p < 0.05$ (Welsh T test). These data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series Accession number GSE11826.

Semi-quantitative Real Time RT-PCR

Relative levels of message expression of *Cirbp*, *Mt1* and *2*, *Phlda3*, and *Ccng* were performed by RT-PCR. Equal amounts of total RNA from right forelimb mesoderm were primed with Oligo dT and reverse transcribed using Superscript II (Invitrogen). Samples were PCR amplified in the presence of SyberGreen (Quantitect Sybergreen PCR kit, Qiagen, Valencia, CA) using gene-specific primers. Melting curves and gel electrophoresis were examined to confirm specificity of product amplification. Chip array data and preliminary experiments indicated that α-tubulin mRNA levels were unchanged by acetazolamide or cadmium exposure and thus was used to normalize the semi-quantitative PCR data. The PCR amplifications and monitoring of product generation per cycle were performed using a DNA Engine Opticon 2 Continuous Fluorescence Detector and evaluated using its software. Gene expression levels were determined by gene-specific standard curves in the linear amplification phase. All values were normalized to tubulin within the same sample.

RESULTS

Teratogen-Induced Postaxial Forelimb Ectrodactyly

In a previous manuscript (Scott et al., 2005) we reported that cadmium sulfate, 4 mg/kg given i.p. on E9.5 to C57 BL/6 mice, induced postaxial ectrodactyly of the right forelimb in 77% of fetuses examined at E18.5. For the present work we derived a new regimen for acetazolamide: i.p. administration of a water-insoluble form of acetazolamide (Sigma), which was suspended in vegetable oil for maternal injection. A dose of 400 mg/kg given at E9.5 induced postaxial right forelimb ectrodactyly in 42 of 54 live fetuses, a frequency of 78%. Thus both regimens of teratogen exposure induce a very similar frequency of postaxial right forelimb ectrodactyly and thereby represent a good comparison to search for gene expression changes relevant to ectrodactyly induction.

Microarray Analysis

Limb bud ectoderm

3 hr ectoderm. There were 15 genes whose expression level was changed more than twofold in response to acetazolamide (Table 1); invariably the expression level always was increased. In relation to our central hypothesis, a number of these genes have been reported to exhibit increased expression in response to a variety of cellular stressors: for example, *Anp32A* (Gallouzi et al., 2001), *Clcn3* (Comes et al., 2005), *Wnk1* (Lenertz et al., 2005), *Abca1* (Kaminski et al., 2006), *Nrdgl* (Agarwala et al., 2000), *Sdc4* (Oh and Couchman, 2004), *Map3k1* (Zhang et al., 2005), *Cirbp* (Fujita, 1999), and *Ppm1b* (Baines and Molkentin, 2005). However, there is little

Table 1
Genes Misregulated ≥ Twofold in 3 Hr Teratogen-Exposed Limb Bud Ectoderm

Affymetrix probe set ID	Gene symbol	Gene title	Fold change	Intensity of expression
Acetazolamide				
93372_at	Anp32a	Acidic (leucine-rich) nuclear phosphoprotein	4.2	M
94354_at	Abca1	Cholesterol efflux regulatory protein	2.8	L
94464_at	Clcn3	Chloride channel 3	2.8	L
94465_at	—	—	1.9	L
161270_i_at	Wnk1	Protein kinase, lysine-deficient 1	2.6	L
95387_f_at	Sema4b	Semaphorin 4b	2.6	M
96596_at	Nrdgl	N-myc down-regulated gene 1	2.5	M
160464_s_at	—	—	2.2	M
98590_at	Sdc4	Syndecan 4	2.4	M
100706_f_at	Sfmbt2	Sex comb. midleg-like with 4MBT domains	2.4	L
103020_s_at	Map3k1	Mitogen-activated protein 3 kinase 1; MEKK1	2.4	M
103506_f_at	Dsc2	Desmocollin 2	2.3	M
93284_at	Cirbp	Cold-induced RNA binding protein	2.2	M
96749_f_at	Tmem30b	Transmembrane protein 30b	2.2	L
96748_at	—	—	1.8	L
100475_at	Trim25	Estrogen-responsive finger protein	2.1	M
101836_at	Ppm1b	Protein phosphatase	2.1	L
102768_i_at	Sc5d	Sterol C5 desaturase	2.0	M
102769_f_at	—	—	1.9	M
Cadmium				
103925_at	Mllt3	Myeloid/lymphoid or mixed lineage Leutranslocation to 3 homolog	0.5	H

Intensity of expression was arbitrarily assigned as low (L) if the normalized score was less than 500; assigned as medium (M) if the score was 500–5000; and assigned as high (H) if the score was greater than 5000.

obvious commonality in the type of stress response (e.g., enotoxic stress, ER stress, heat shock response) in which these genes are known to be involved, and none that has been linked to lowered Shh signaling.

The gene exhibiting the greatest change of expression (up 4.2×) encodes an acidic nuclear protein, Anp32A (also known as Lamp, pp32, Phap1, mapmodulin). This gene is the founding member of a family of leucine-rich acidic nuclear proteins that possess a carboxyterminal acidic tail (Matilla and Radrizzani, 2005). These features provide the basis for binding of Anp32A to numerous proteins, thereby having functional consequence in many important cellular signaling pathways, many of which are integral to vertebrate limb morphogenesis. Thus Anp32A influences MapK/ERK signaling as an inhibitor of Pp2A (Yu et al., 2004; Fukukawa et al., 2005) and Wnt signaling via interaction with Axin (Stelzl et al., 2005). Other potentially important interactions include repression of histone acetylation (Seo et al., 2002; Kutney et al., 2004; Schneider et al., 2004; Fan et al., 2006) binding to NCAM (Buttner et al., 2005) and interaction with the DNA binding domain of the estrogen receptor (Era) and other nuclear hormone receptors (Loven et al., 2004) to modulate transcription of hormone-responsive genes. It is of great interest that Anp32A also functions to regulate export of some mRNAs through interaction with the nuclear export factor CRM1 and by virtue of binding to HUR, a member of the embryonic lethal abnormal vision (ELAV) family of RNA binding proteins. These ELAV proteins bind to AU-rich elements in the 3′UTR that target the mRNAs for rapid degradation; and this interaction of HUR with Anp32A is strengthened by cellular stress such as heat shock (Gallouzi et al., 2001).

A second potential mRNA processive gene, cold-inducible RNA-binding protein (*Cirbp*), was increased in the limb bud ectoderm more than two times after 3 hr exposure to acetazolamide. This gene, also known as A18hnRNP, encodes a protein involved in mRNA stability especially of stress responsive genes (Yang and Carrier, 2001), possibly by binding HuR (Aoki et al., 2003). The expression of *Cirbp* was also increased in the limb bud mesoderm 3 hr after exposure to acetazolamide (1.7-fold up) or cadmium (1.8-fold up), and this increase was confirmed by RT-PCR (Fig. 1a). Of parallel interest, *Cirbp* expression was increased in whole embryo microarray preparations after 5 hr exposure to cadmium (Kultima et al., 2006). This increase was not confirmed by RT-PCR, but embryos were collected 10 hr after cadmium injection. Our own microarray results indicated unchanged *Cirbp* expression levels in both limb bud tissues after 12 hr exposure to cadmium or acetazolamide, suggesting that increased *Cirbp* expression may be a common early response to embryonic stress.

Although *n-myc* mRNA was not demonstrably downregulated in the microarray, the results in Table 1 indicate that exposure to acetazolamide leads to a lowering of N-myc signaling in the limb bud ectoderm. This can be most readily visualized by an increased expression of *Ndr1* (N-myc downregulated gene 1; also known as *Ndrg1*, *Drg1*, *Cap43*, *RTP/rit42*, and *Proxy1*) of approximately 2.5-fold as indicated by two different oligonucleotide probes. In support of lowered N-myc signaling two other genes from Table 1 are regulated by myc signaling, *MAP3K1* and *Ccln3* (myc target gene database, http://www.mycancergene.org/site/mycTargetDB.asp); and MAP3k1, which was demonstrated to be down-regulated by c-myc (Qingbin et al., 2000), was up-regulated in limb bud ectoderm suggestive of lowered N-myc signaling. Because *N-myc* is a direct target of Shh signaling (Oliver et al., 2003) these results support the concept that teratogen exposure does lead to lowered Shh signaling and promote the concept (Bell et al., 2005) that Shh signaling in the limb bud ''ectoderm'' may have an important role in limb morphogenesis.

Nrd1 is a 43kDa protein belonging to a family with four members, none of which has a clearly identified function. Expression of this gene is upregulated during cellular differentiation by a variety of chemical agents, hypoxia, and DNA damage in p53-dependent manner (Kurdistani et al., 1998). *Nrd1* is expressed in the early mouse limb bud at sites of n-myc expression; and lowering of n-myc signaling does result in an increased expression of *Nrd1* (Shimono et al., 1999).

As might be expected from the inhibition of carbonic anhydrase by acetazolamide, there is indication of ionic imbalance by the up-regulation of *Clcn3* and *Prkwnk1* (Table 1). Clcn3 is an intracellular chloride channel (or Cl^-/H^+ exchanger) found on vesicles of the endocytic and lysosomal pathways to facilitate endosomal acidification (Stobrawa et al., 2001; Hara-Chikuma et al., 2005). This chloride channel is gated by many factors, including intracellular pH, and has been functionally associated with cell volume regulation, proliferation, and apoptosis (Guan et al., 2006). Prkwnk1 (*Wnk1*) is a serine/threonine kinase from a family of four that have been shown to regulate ion permeability in epithelia, and *Wnk1* expression in the adult animal is localized in polarized chloride transporting epithelia (Choate et al., 2003). This activity, often in conjunction with other family members (Lenertz et al., 2005), is accomplished by regulation of various transporters (Yang et al., 2003; Xu et al., 2005; Vitari et al., 2006).

An interesting feature from Table 1 is the presence of two genes involved in cholesterol metabolism, *Abca1* and *Sc5d*. Abca1 belongs to a highly conserved multispan protein family that facilitates the movement of defined substrates across cell membranes (Kaminski et al., 2006). The major function of the Abca1 protein is to aid in the cellular efflux of cholesterol, although it can function in the export of other molecules such as annexin1 (Omer et al., 2006). Recently it has been shown that ABCA1 can control the distribution of cholesterol and sphingomyelins from lipid raft to non-raft microdomains of the plasma membrane (Landry et al., 2006). Such redistribution could affect SHH signaling as lipid rafts are thought to be important in this pathway (Karpen et al., 2001). Sc5d is part of the distal biosynthetic cholesterol pathway where the protein product catalyzes the conversion of lathosterol to 7-dehydrocholesterol. Interestingly, Sc5d deficiency leads to *postaxial* polydactyly in mice and humans, a response similar to that seen in children with Smith-Lemli-Opitz syndrome (SLOS) (Krakowiak et al., 2003). SLOS is due to deficiency of DHCR7, which converts 7-dehydrocholesterol to cholesterol. The presence of postaxial polydactyly in children with deficiency of DHCR7 or Sc5d could be viewed as the functional opposite of postaxial ectrodactyly due to excessive Sc5d as seen here in the limb bud ectoderm after 3 hr exposure to acetazolamide. This concept bears continued scrutiny because DHCR7 has now been shown to have a role as a

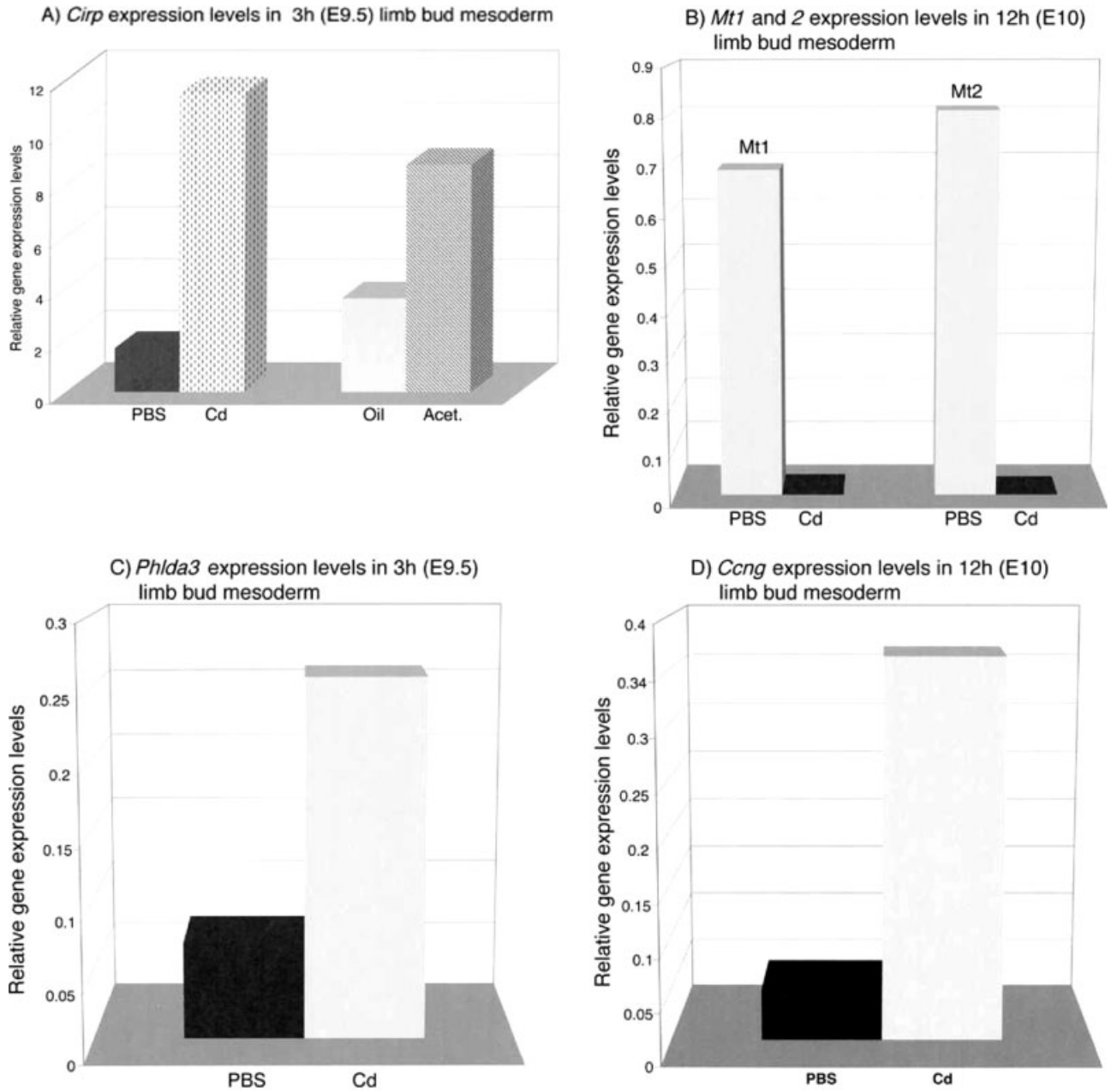

Figure 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) quantitation of the embryonic day 9 or 10 limb bud mesoderm.

negative regulator of Hh signaling (Koide et al., 2006) in contrast and in addition to its more common role as a positive Hh signaling regulator. Moreover, the substrate of this enzyme, 7-dihydrocholesterol, is a known precursor of vitamin D3 and has recently been considered as the ''small chemical agent'' translocated by Ptc to repress Smo and thus down-regulate Hh signaling (Bijlsma et al., 2006).

Interestingly, there was only a single gene whose expression was changed sufficiently, because of cadmium exposure, for inclusion in Table 1. The expression of *Mllt3*, a Drosophila trithorax homolog, was reduced twofold in the limb bud ectoderm after 3 hr exposure to cadmium sulfate. Thus we could find no obvious common effect on gene expression by these two teratogens in this limb tissue at this time interval after administration. This same phenomenon, that is, little similarity in gene expression profile by agents that induce the same abnormal phenotype, was reported by Kultina et al. (2006), studying cadmium and valproic-acid–induced exencephaly.

12 hr ectoderm. Monitoring gene expression in stage 2.5 forelimb bud ectoderm revealed 37 annotated genes whose expression was changed at least twofold after 12 h exposure to acetazolamide (Table 2). Once again, all changes in gene expression were up-regulation.

Table 2
Genes Misregulated ≥ Twofold in 12 Hr Acetazolamide-Exposed Limb Bud Ectoderm

Affymetrix probe set ID	Gene symbol	Gene title	Fold change	Intensity of expression
161270_i_at	Wnk1	Protein kinase, lysine-deficient 1	4.7	L
160739_at	—	—	2.8	L
94464_at	Clcn3	Chloride channel 3	4.2	L
94465_g_at	—	—	2.0	L
97808_at	Sf3B1	Splicing factor 3B	3.4	L
101104_at	Sbno	Strawberry notch homolog 1	3.4	M
97504_at	Ccnd2	Cyclin D2	3.2	M
103611_at	CD47	CD47 antigen/Integrin-associated protein	3.2	L
102062_at	Smarcc1	Swi/Snf-related regulator of chromatin C1	3.0	M
94689_at	Rbm39	RNA binding motif protein 39/Caper	3.0	L
101451_at	Peg3	Paternally expressed gene 3	3.0	M
102348_at	Xdh	Xanthine dehydrogenase	2.9	L
101441_i_at	Itpr2	Inositol 1, 4,5 triphosphate receptor 2	2.9	H
93246_at	Narg1	NMDA receptor-regulated gene 1/tubedown	2.8	L
100499_at	Stx3	Syntaxin 3	2.6	M
103789_at	Brd4	Bromodomain-containing protein 4	2.6	M
99042_s_at	Shox2	Short stature homeobox 2	2.6	L
160696_at	Tia1	Cytotoxic granule-associated RNA Binding protein 1	2.4	L
93169_at	Zfp322a	Zinc finger protein 322a	2.4	M
93367_at	Fbxw7	F-box and WQ-40 domain protein 7 (archipelago homolog)	2.4	L
93964_s_at	Ddx6	DEAD box polypeptide 6	2.3	L
93965_r_at	—	—	2.0	L
100501_at	Iqgap	IQ motif containing GTPase activating protein 1	2.3	L
101787_f_at	Ccrn4	Carbon catabolite repression 4-like/nocturnin	2.3	M
95092_at	Ppp3ca	Calcineurin	2.3	M
101542_f_at	Fin14/Ddx3x	Fibroblast growth factor inducible 14/DEAD box polypeptide 3, X linked	2.2	M
100457_at	Glg1	Golgi apparatus protein 1	2.2	M
94364_at			2.2	M
103421_at	Sdfr2/Frrs1	Stromal-cell–derived factor receptor 2/ferric-chelate reductase	2.2	L
92888_s_at	Styx	Phosphoserine/threonine/tyrosine PO_4ase	2.2	L
103064_at	Rala	v-ral simian leukemia viral oncogene homolog A	2.2	M
100713_at	Zfp617	Zinc finger protein 617	2.1	L
102224_at	Igf1r	Hypothetical protein D930020L01 (insulin-like growth factor 1 receptor)	2.1	M
103288_at	Nrip	Nuclear receptor interacting protein	2.1	L
102893_at	Pou2f1	POU domain class 2, transcription factor 1/Oct1	2.1	L
94852_at	Glul	Glutamate-ammonia ligase (glutamine synthase)	2.1	M
99665_at	Satb1	Special AT-rich sequence binding protein 1	2.1	L
103079_at	Arid2	AT-rich interactive domain 2	2.1	M
96920_at	Htra1	Protease, serine, 11	2.1	M
97848_at	Rbmx	RNA binding motif protein, X chromosome	2.0	M
93464_at	Akap9	A kinase anchor protein/Yotiao	2.0	L
Nonannotated genes				
103082_at		Riken cDNA E230022H04	3.1	M
98849_at		DNA segment, Chr 8, BWG1414e	2.0	M

As regards the hypothesis of stress response as the inducer of lowered Shh signaling leading to subsequent postaxial ectrodactyly, there is little support from these data. There is some indication that p53 signaling is upregulated in 12 hr acetazolamide-exposed limb bud ectoderm; Peg3 and Fbxw7 are p53-regulated genes showing a >2× increase in expression; and a few others, Mdm4, Gas2, and Wig1 (Zmat), were increased but not to a level of twofold. These observations must be tempered by the lack of increased expression of ''typically'' responding p53 genes such as p21 and Ccng1.

Xdh, a gene involved in oxidative stress, showed increased ectodermal expression, 1.88-fold 3 hr after acetazolamide exposure, and continued to increase with time being elevated threefold by 12 hr.

As at the 3 hr interval, several RNA ''processing'' genes were up-regulated 2× or more in the 12 hr acetazolamide limb ectoderm. These included Sf3b1, Rnpc2, Tia1, Rbmx, Ddx3x, Ddx6, Ccrn4l (nocturnin), Styx, and Sbno (Table 2). Interestingly there is little similarity in the genes up-regulated at the two intervals except that Rnpc2 was increased 1.88-fold at 3 hr (vs. 3.0-fold at 12 hr), and *Hnrpa*

was up-regulated in the 12 ectoderm, $p < 0.0138$ (vs. 1.74 at 3 hr). The finding of up-regulated mRNA ''processing'' genes is of interest because a number of genes sharing this classification were shown to be positive regulators of Hh signaling (Nybakken et al., 2005). We interpret the increase of mRNA ''processing'' genes to be a precursor of restoring Hh signaling, which is severely compromised in acetazolamide exposed mouse limb buds (Bell et al., 2005).

Another functional classification, chromatin remodeling, had many genes with elevated expression in limb bud ectoderm 12 hr after exposure to acetazolamide. These included *Smarcc1*, *Brd4*, *Nrip2*, *Satb1*, and *Arid2* (Table 2).

The two most highly up-regulated genes in the acetazolamide 12 hr ectoderm, *Wnk1* (up 4.7-fold) and *Clcn3* (up 4.2-fold), are both involved in ionic regulation (Gamba, 2005; Jentsch et al., 2005; Suh and Yuspa, 2005). Both of these genes were also up-regulated in 3 hr acetazolamide ectoderm: *Clcn* (up 2.8×), *Wnk* (up 2.6×).

In the limb bud ectoderm 12 hr after exposure to cadmium sulfate there were 32 annotated genes whose expression was changed twofold or greater (Table 3). Seven of these increased, led by Cyclin g1 (up 3.5-fold) and *Cdkn1a-p21* (up 2.5-fold). Both of these genes are inducible by p53, whose activity is reportedly increased after cadmium exposure, including the mouse embryo (Fernåndez et al., 2003; Kultima et al., 2006); the effect of increasing *Ccng* and *p21* would presumably restrict proliferative activity in the limb bud ectoderm. Of the five remaining up-regulated genes, three are associated with

Table 3
Genes Misregulated ≥ Twofold in 12 Hr Cadmium-Exposed Limb Bud Ectoderm

Affymetrix probe set ID	Gene symbol	Gene title	Fold change	Intensity of expression
160127_at	Ccng1	Cyclin g1	3.5	M
98067_at	Cdkn1a	p21, cyclin-dependent kinase inhibitor	2.5	M
94881_at	—	—	1.8	M
93903_at	Acvr2b	Activin receptor 2b	2.4	M
160458_at	Mcam	Melanoma adhesion molecule	2.1	L
96749_at	Tm30b	Transmembrane protein 30b	2.0	L
100009_r_at	Sox2	Sry box 2	2.0	L
100596_at	Selenbp1	Selenium binding protein 1	2.0	M
99467_at	Rasa1	Ras p21 protein activator	0.50	M
93257_at	Ddx1	DEAD box polypeptide 1	0.50	H
92190_at	Nr2c1	Nuclear receptor subfamily 2, group c, member 1	0.50	M
95318_at	Zfp105	Zinc finger protein 105	0.50	H
99575_at	Ubqln1	Ubiquilin 1	0.49	H
97893_at	Tbpl1	TATA box binding protein-like 1	0.49	M
101370_at	Kpna1	Karyopherin (importin) alpha 1	0.48	M
94209_g_at	Txndc7	Thioredoxin domain containing 7 protein/ Pdia6 disulfide isomerase family A, member 6	0.48	H
99963_at	Zfp101	Zinc finger protein 101	0.48	M
102000_f_at	Uqcrc2	Ubiquinol cytochrome C reductase core protein 2	0.47	H
103674_f_at	eIF2s3y	Eukaryotic translation initiation factor, subunit 3	0.47	M
104711_at	Vps4a	Vacuolar protein sorting 4a	0.47	M
92503_at	Hus1	Hydroxyurea-sensitive 1	0.46	M
96711_at	Znrd1	Zinc ribbon domain containing 1	0.46	M
104582_g_at	Zdhhc6	Zinc finger, DHHC domain containing 6	0.46	M
104583_at			0.38	M
93089_at	eIF4a2	Eukaryotic translation initiation factor 4A2 (Ddx2B)	0.45	H
160423_at	Mrps2	Mitochondrial ribosomal protein S2	0.43	M
101002_at	Azin1	Ornithine decarboxylase antizyme inhibitor	0.42	H
104604_at	Zfp96	Zinc finger protein 96	0.39	M
100892_at	Ndufaf1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	0.38	M
93567_at	Pfn2	Profilin 2	0.37	M
101561_at	Mt2	Metallothionein 2	0.36	H
98423_at	Gjb2	Gap junction membrane channel protein beta 2, Cx26	0.35	M
99365_at	Cq3	Coenzyme Q3 homolog, methyltransferase, ubiquinone biosynthesis	0.34	M
160900_at	Gkap	G kinase anchoring protein 1 large associated protein	0.31	M
Nonannotated genes				
100465_i_at		Clone IMAGE:3371572, mRNA	2.0	M
103082_at		Riken cDNA E230022H04	0.5	M
160595_at		Riken cDNA 2310042P20, TPR repeat, transferase	0.5	M
104142_at		Riken cDNA 2810013E07, cyclin E2?	0.49	M
93055_at		Riken cDNA 1110054N06, ankyrin repeat	0.49	M
98464_at		Riken cDNA 1110011C06 ankyrin repeat domain 40	0.48	M
160885_at		Riken cDNA 2700010L10, CkalphaCdk substrate	0.47	H
104327_at		Riken cDNA 9030612M12, zinc fingers	0.40	M

cell polarization/cell migration (*Mcam*, *Tmem30b*, *Selbp*); *Acvr2b* is integral to left/right patterning of the viscera, and *Sox2* is a transcription factor involved in numerous developmental processes and has been implicated in Hh, Wnt, and Fgf signaling. There is no overlap with the large collection of genes up-regulated in the acetazolamide-exposed 12 hr limb bud ectoderm. The data suggest a reversal of gene expression relative to cell proliferation, a restrictive profile in cadmium-exposed limbs, that is, up-regulated *Ccng1* and *p21*, versus a stimulatory profile in acetazolamide-exposed limbs, that is, up-regulated *Ccnd2*. This dichotomy is epitomized by the dysregulation of a nonannotated gene, E23022H04, up-regulated 3× in acetazolamide 12 hr limb bud ectoderm, but down-regulated 2× by cadmium (Table 2). Another noteworthy finding was the ''down-regulation'' of metallothionein2 (Mt2) expression. Similar changes in Mt gene expression were also noted in the cadmium-exposed limb bud mesoderm where an extended comment about plausible cause and possible effect will be discussed.

Limb bud mesoderm. In stark contrast to the teratogen-exposed limb bud ectoderm, the underlying mesenchyme exhibited very few genes whose expression was altered twofold or greater (Table 4).

3 hr mesoderm. After 3 hr exposure to acetazolamide, the expression of four genes (three up-, one down-regulated) was changed twofold or greater. There were no genes whose expression was changed to this extent by exposure to cadmium.

12 hr mesoderm.. After 12h exposure to acetazolamide, there were no genes in which expression was changed twofold or greater. However, there were 12 genes (four up-, eight down-regulated) that met the imposed criteria, after 12 hr exposure to cadmium. As in the cadmium-exposed limb bud ectoderm, the most changed genes were *Ccng1* (confirmed by qPCR to be elevated ca. 4.5×, Fig. 1) and *p21*, p53-inducible and generally functioning to restrict cell proliferation. A surprising finding was a very significant reduction, approximately sevenfold, in the expression of *Mt2*. Heavy metals such as cadmium routinely increase Mt levels transcriptionally, presumably as a mechanism to sequester the toxin, thereby protecting the organism. We thus examined the microarray results of the other murine *Mts*, 1, 3, and 4; *Mts* 3 and 4 were essentially not present in limb ectoderm or mesoderm in the 3- or 12-hr control samples, and were not induced in either tissue by cadmium or acetazolamide. *Mt1*, like *Mt2*, was very substantially reduced after 12 hr of cadmium exposure in the limb bud mesoderm. We reiterate here that *Mt2* mRNA was significantly reduced in the limb bud ectoderm after 12 hr exposure to cadmium (Table 3); *Mt1* mRNA in this tissue was slightly lower than in corresponding controls, but not significantly by the criteria employed in these analyses. *Mt 1* and 2 were unchanged from controls, 3 hr after exposure to either teratogen, and there were no changes attributable to acetazolamide after 12 hr exposure in limb ectoderm or mesoderm.

We also examined the data regarding *Mtfs* (metal response element-binding transcription factor) because of their role in cadmium-induced metallothionein transcription. *Mtf1*, the primary gene for cadmium-induced metallothionein transcription, was considered absent from all control and teratogen-exposed samples (ectoderm and mesoderm) at 3 and 12 hr. This result is consistent with the *Mtf* null phenotype. $Mtf^{-/-}$ embryos are macroscopically normal at E13.5 (Gunes et al., 1998), suggesting this transcription factor is not needed at earlier embryonic stages. A second Mtf, *Mtf2/Zirf1* (Remondelli and Leone, 1997), represented by three different probes, was detectable in both limb tissues, at both times in control and teratogen-exposed samples. There was little change from

Table 4
Genes Misregulated ≥ Twofold in Teratogen-Exposed Limb Bud Mesoderm

Affymetrix probe set ID	Gene symbol	Gene title	Fold change	Intensity of expression
3 hr-Acetazolamide				
95913_at	B230333c21	RNA binding region protein	2.3	M
100405_at	Cbx3	Chromobox homolog 3	2.2	M
160708_at	Schip1	Schwannomin interacting protein	2.1	M
95379_at	Mab21l2	Male-abnormal 21 (*C. elegans*) like 2	0.48	M
3-hr cadmium				
None				
12-hr acetazolamide				
None				
12-hr cadmium				
94881_at	Cdkn1a	p21, cyclin-dependent kinase inhibitor 1a	6.0	A[a]
160127_at	Ccng1	Cyclin G1	3.8	L
98056_at	Phlda3	Pleckstrin homology-like domain A3	2.9	M
97834_g_at	Pfkp	Phosphofructokinase, platelet	2.4	L
94924_at	Prrg2	Proline-rich Gla polypeptide 2	2.0	M
92558_at	Vcam1	Vascular cell adhesion molecule	0.49	M
94818_ar	0gt	0-linked N-acetylglucosamine transferase	0.47	M
92503_at	Hus1	Hydroxyurea-sensitive 1	0.39	M
101561_at	Mt2	Metallothionein 2	0.14	H
Nonannotated genes				
160966_at		cDNA clone MGC: 96550 IMAGE: 30547278 Zpf 187	0.48	M
97816_at		Riken cDNA 2600011c06, RNA splicing	0.48	M
94459_at		Hypothetical LOC225897, mRNA	0.41	M

[a]Absent.

controls in *Mtf2* mRNA levels in 3 hr teratogen-exposed samples and in 12 hr acetazolamide-exposed samples. However, *Mtf2* appeared down-regulated in 12 hr cadmium-exposed mesoderm manifested by all three probes, but the characteristics of down-regulation did not allow inclusion in Table 3.

We attempted to confirm this microarray-based indication of lowered *Mt1* and *2* transcription using RT-PCR. As shown in Figure 1, the unexpected phenomenon of lowered *Mt1* and *Mt2* mRNA was fully confirmed; in fact, the severity of decrease appears even greater using this technology.

A plausible explanation of this phenomenon is an acute lowering of ''free'' zinc in the embryo due to metallothionein induction in the mother and extraembryonic membranes in routine response to cadmium exposure (Daston et al., 1994; Taubeneck et al., 1994; Bui et al., 1998) coupled with little or no transport of cadmium into the embryo (Dencker, 1975). This series of events would lead to a temporary zinc deficiency within the embryo, a status that could have negative consequences based on numerous zinc metalloproteins with important roles in developmental processes. One of these proteins is *Shh* > (Tanaka Hall et al., 1995), whose dysfunction we believe underlies the induction of postaxial forelimb ectrodactyly.

The role of zinc in *Shh* function is unclear. There is evidence that *Shh* does not act enzymatically as a zinc hydrolase (Day et al., 1999; Fuse et al., 1999), a role zinc often plays in other enzymes. However, mutation of the zinc-coordinating residues leads to loss of zinc from the *Shh* protein, loss of signaling activity, and structural instability (Day et al., 1999). Interestingly, acetazolamide-induced postaxial forelimb ectrodactyly is heavily dependent on zinc status. Lowering dietary zinc increases the frequency of offspring with postaxial forelimb ectrodactyly at a constant dose of acetazolamide (Hackman and Hurley, 1983). Acetazolamide is a sulfonamide that acts pharmacologically through inhibition of carbonic anhydrase, a zinc metalloenzyme. The sulfonamide group of acetazolamide binds to the zinc ion in the active site of carbonic anhydrase, and the affinity of acetazolamide for carbonic anhydrase is decreased by mutation of the zinc-coordinating amino acid residues (Kiefer and Fierke, 1994). The similarity of zinc coordination in carbonic anhydrase to that in *Shh* suggests that acetazolamide, especially at the high doses required to induce teratogenesis, could act directly to perturb hedgehog signaling by binding to the zinc core of *Shh*, thereby leading to postaxial forelimb ectrodactyly.

Another agent that induces postaxial forelimb ectrodactyly, ethanol (Webster et al., 1983; Zimmerman et al., 1990; Kotch et al., 1992), may also act via this mechanism. Ethanol has been shown to induce metallothionein transcription in the murine embryo (Green et al., 2007), the teratogenic effects are substantially lower in metallothionein null mice (Carey et al., 2000), and zinc supplementation can ameliorate ethanol teratogenicity (Carey et al., 2003).

A fourth agent that induces postaxial forelimb ectrodactyly is also implicated in perturbed zinc homeostasis. Valproic acid increases the level of metallothionein in maternal mice and rats (Keen et al., 1989; Kaji and Mikawa, 1991; Bui et al., 1998), yet also induces *Mt1* and 2 transcription in the embryo (Kultima et al., 2004), presumably making the availability of zinc for embryonic utilization even lower. All four of these teratogens most effectively induce postaxial forelimb ectrodactyly when given on day 9 of murine gestation. It is just at this stage that the embryo begins to express Shh in the posterior forelimb mesenchyme. Perhaps the process of incorporating zinc in newly synthesized hedgehog protein, a process that is not well understood (Krezel et al., 2007), is especially sensitive to zinc status. Alternatively, there are hundreds of other zinc-containing proteins, many in the hedgehog signaling pathway (i.e., Gli zinc finger transcriptional regulators), which might also have perturbed function in a zinc-starved environment.

CONCLUSIONS

The goal of the work presented here was to identify a pathway, presumably a stress response pathway, by which two structurally dissimilar teratogens could induce a similar dysmorphology, postaxial right-sided forelimb ectrodactyly. Furthermore it was assumed that perturbation of this pathway would lead directly or indirectly to down-regulation of Hh signaling for reasons enumerated in the Introduction. We expected this pathway would be present in the embryo and perturbed at that site by teratogen exposure.

It seems plausible to consider that cadmium may work by this mechanism except that the stress response pathway, that is, induction of metallothionein, is located in the mother and extra embryonic membranes. The concept that teratogen administration could act to limit zinc levels or activity within the embryo and thereby induce abnormal development has solid scientific support from previous studies (Daston et al., 1994; Taubeneck et al., 1994; Bui et al., 1998; Kultima et al., 2004; Lee et al., 2004; Fernåndez et al., 2007). We contribute indirect evidence in support of this general concept and suggest a novel mechanism by which lowered embryonic zinc concentration could be translated into abnormal embryogenesis, that is, posterior forelimb ectrodactyly.

We are intrigued by the potential role of zinc status as a mechanism of teratogen action. *Shh* is a zinc-containing protein; the role of zinc in *Shh* signaling is thought to be structural, not catalytic (Fuse et al., 1999), and the mutation of zinc coordination sites leads to loss of signaling activity (Day et al., 1999). Four teratogens, which induce postaxial right-sided forelimb ectrodactyly, acetazolamide, cadmium, ethanol, and valproic acid, are known or thought to affect zinc status in the rodent embryo. Moreover, mutation of zinc coordination site residues or their close neighbors have been associated with human holoprosencephaly, a loss of Hh signaling activity, and the absence of *Shh-N* peptide (Traiffort et al., 2004). These threads of preliminary information should lead to more definitive experimentation regarding the role of zinc in *Shh* function and whether perturbation of this role can lead to abnormal development.

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