Adriamycin-Induced Models of VACTERL Association

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Abstract

Animal models are of great importance for medical research. They have enabled analysis of the aetiology and pathogenesis of complex congenital malformations and have also led to major advances in the surgical and therapeutic management of these conditions. Animal models allow us to comprehend the morphological and molecular basis of disease and consequently to discover novel approaches for both surgical and medical therapy. The anthracycline antibiotic adriamycin was incidentally found to have teratogenic effects on rats, producing a range of defects remarkably similar to the VACTERL association of congenital anomalies in humans, providing a reproducible animal model of this condition. VACTERL association is a spectrum of birth defects which includes vertebral, anal, cardiovascular, tracheo-oesophageal, renal and limb anomalies. In recent years, adriamycin rodent models of VACTERL have provided valuable insights into the pathogenesis of this complex association, particularly in relation to tracheo-oesophageal malformations. The adriamycin rat model and adriamycin mouse model are now well established in the investigation of the morphology of faulty organogenesis and the regulation of gene expression in tracheo-oesophageal anomalies.

Congenital anomalies such as oesophageal atresia/tracheo-oesophageal fistula (OA/TOF) are relatively common and require urgent surgical intervention soon after birth. The causation and pathogenesis of these complex malformations are poorly understood. Scrutinising aetiological factors is difficult in the early embryological stages when these defects arise but can have significant rewards. In an era of rapid expansion in the field of genetic research, novel therapies and genetic screening for congenital anomalies would be potentiated by the outcomes of these enquiries. Examination of early developmental gene disturbance and structural abnormalities has been restricted by the inability to conduct studies on embryonic human materials. To this end, animal models have enabled advances in congenital malformation research. A constellation of anomalies, including gastrointestinal, bone, renal, and cardiovascular defects were fortuitously discovered in the offspring of treated pregnant rats during teratogenicity studies for the anticancer drug adriamycin [Thompson et al., 1978]. This phenotype was remarkably similar to the VACTERL association of congenital malformations in humans and included OA/TOF-like anomalies. These findings have been exploited to establish important animal models of OA/TOF, the adriamycin mouse model (AMM) and the adriamycin rat model (ARM). The purpose of this review is to synthesise the evidence for developmental origins of OA/TOF and VACTERL derived from the ARM and AMM.

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Oesophageal Atresia and the VACTERL Association

OA/TOF occurs in approximately 1:3,500 newborns every year [Depaepe et al., 1993]. The term OA/TOF encompasses a variety of tracheo-oesophageal malformations of obscure aetiology which, despite the comparative frequency with which they arise, remain highly complex to manage both medically and surgically. Typically, the upper oesophagus is blind ending and an anomalous connection exists between the trachea and the stomach, the fistula. This life-threatening condition requires immediate surgery at birth to correct the anatomy. Despite survival rates benefitting immensely from advances in surgical technique and neonatal care, long-term morbidity is problematic [Kovesi and Rubin, 2004]. Chronic respiratory problems, oesophageal dysmotility and gastro-oesophageal reflux disease (GORD) often confer poor quality of life on affected children postoperatively [Spitz, 2007]. Some of these problems may be sequelae of surgical intervention; however, many hypothesise that there is an inherent inability of the malformed lower oesophagus to fulfil a functional role.

OA/TOF arises in isolation only 50% of the time; the remainder of cases present with other defects, most frequently with the VACTERL association of congenital anomalies [Pedersen et al., 2012]. VACTERL occurs in up to 1:10,000 neonates per year [Botto et al., 1997] and is defined by the occurrence of at least 3 of Vertebral, Anorectal, Cardiovascular, Tracheal, oEsophageal, Renal and Limb defects simultaneously [Solomon, 2011]. A minority of OA/TOF cases arise in syndromes with known genetic background, for example CHARGE syndrome, Feingold syndrome and Fanconi anaemia [Shaw-Smith, 2006]. However, no unifying aetiological factor has been identified for OA/TOF occurring in isolation or along with VACTERL. The low rate of familial recurrence suggests that monogenetic causation is unlikely [Brunner and van Bokhoven, 2005]. VACTERL is classified as an association, defined by Lubinksy [1986] as 'derivatives of nonspecific teratologic events acting on developmental fields'. These defects coalesce in a nonrandom fashion, and single or multiple diffuse insults may affect many organ anlagen during a critical window in early development. The nature of the insult in VACTERL and the mechanism by which it induces a variety of tissue anomalies remains unknown. Progress to uncover these mechanisms is deterred by an inadequate understanding of the normal developmental process by which the oesophagus forms.

What we know of normal embryological development in humans is limited and somewhat historical [Gray and

Skandalakis, 1972], and the most up-to-date information arises from animal studies, particularly in the mouse. The oesophagus and trachea share a common embryological predecessor, the foregut. A series of complex morphological manoeuvres coordinated by many interacting signalling pathways are responsible for the development of 2 anatomically and functionally distinct structures from a single precursor. During gastrulation, cells in the embryo organise into 3 layers, the endoderm, mesoderm and ectoderm, in preparation for impending massive re-configuration. The endoderm undergoes extensive folding to form a tube, the cranial end of which becomes the foregut, the midgut and hindgut arising caudally [Wells and Melton, 1999]. The foregut undergoes a process of separation generating respiratory structures ventrally and the oesophagus dorsally. In humans, foregut separation occurs during the 3rd through 7th weeks of gestation, corresponding to embryonic day (E) 9–11.5 in the mouse and E11–13 in the rat.

Patterning signals in the early embryo designate cell fate identity in specific regions of the foregut, directing organogenesis of the thyroid, thymus and lungs from ventral foregut endoderm and the oesophagus from dorsal foregut endoderm [Cardoso and Lu, 2006]. Crosstalk of signals between the foregut endoderm and the surrounding mesoderm is fundamental to the establishment of these precise domains [Masters, 1976; Shannon et al., 1998; Jacobs et al., 2012]. The respiratory primordia, commonly referred to as lung buds, bulge from the ventro-lateral walls of the foregut into the surrounding mesenchyme and elongate, displaying branching morphogenesis. The foregut divides from below the level of the future pharynx, generating a ventral trachea leading to the respiratory structures and a dorsal oesophagus in continuity with the stomach caudally [Perl et al., 2002]. The mechanism by which this division occurs is classically thought to be a process of septation of the lumen by inwardly advancing lateral wall ridges [Gray and Skandalakis, 1972]. It is likely that an amalgam of signalling pathways regulate these anatomical and functional transformations. The vital influencing molecules have not been definitively identified; however, elements of sonic hedgehog (Shh), retinoic acid (RA), bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt pathways are just some which could play significant roles [Grapin-Botton and Constam, 2007; Felix et al., 2009].

In comparison to extensive research into the organogenesis of other foregut-derived structures such as the liver and lung [Maeda et al., 2007; Si-Tayeb et al., 2010], data regarding the modelling of tracheo-oesophageal

structure is found wanting. The physical changes, the cellular mechanisms by which they are achieved and the molecular signals that coordinate these events all require illumination. To this end, investigations in the ARM and AMM have provided some insight into potential disturbances of organogenesis in tracheo-oesophageal malformations.

The Model

The desire to comprehend the basic developmental errors from which congenital malformations arise drives clinicians and developmental biologists to seek ways to examine these conditions in an embryological context. Establishing animal models of congenital anomalies is a valid means to circumvent lack of human research materials [Rosenthal and Brown, 2007]. By identifying or inducing pathology of interest in a small vertebrate with multiple progeny per breeding cycle, short gestation period and low maintenance costs, a feasible vehicle for research purposes can be established. Although an animal model can do no more than mimic the true human condition, the striking phenotypic similarity of the deformities in ARM and AMM to the human VACTERL anomalies make it a particularly appropriate research implement.

Adriamycin is a glycosidic antibiotic of the anthracycline group, isolated from *Streptomyces peucitius*, and is commonly used for treatment of cancers such as sarcoma, lymphoma, leukemia, neuroblastoma, and breast cancer [O'Bryan et al., 1973; Tan et al., 1973; Young et al., 1981]. It enters the nucleus, intercalates into DNA and causes protein-associated single and double stranded DNA breaks [Cullinane et al., 1994]. It is suggested that the major mechanism of adriamycin involves formation of interstrand crosslinks with DNA, interfering with DNA replication and transcription, through inhibiting nucleic acid synthesis [Meriwether and Bachur, 1972; Momparler et al., 1976; Cullinane et al., 2000]. No teratogenic effects have been recorded in the children of mothers who received adriamycin whilst pregnant to date except a single case report of a child with anorectal malformation [Murray et al., 1984]. This may be due to dosage, timing of administration outside the critical window of organogenesis or interspecies differences. Adriamycin has also been used to induce fibrosis-mediated cardiotoxicity and nephropathy models in adult rodents [Fogo, 2003; Gianni et al., 2008]. The molecular influence by which adriamycin induces faulty organogenesis is unknown. Altered apop-

tosis or the production of reactive oxygen species are possible cellular mechanisms of adriamycin action; however, no definitive evidence is available to date [Dunkern and Mueller-Klieser, 1999; Gutteridge and Halliwell, 2000].

Following the inadvertent discovery of a range of defects in the offspring of adriamycin-treated pregnant rats by Thompson et al. [1978], the potential of employing this teratogen to establish an animal model of OA/TOF and VACTERL was not realised until Diez-Pardo et al. [1996] published on the successful production of adriamycin-treated rat embryos with tracheo-oesophageal malformations some 20 years later. The ARM was subsequently reproduced by several groups in a reliable fashion. The first step in creating the model was to identify the response curve between adriamycin dosage and birth defects [Orford and Cass, 1999], and then to pinpoint the crucial window in which treatment would induce the desired defects [Qi et al., 1996]. Sprague Dawley rats were the most commonly used breed in the model, and a range of treatment regimens were used. Defects were successfully produced by daily intraperitoneal injection on E6 through 9 at dose ranges between 1.75 and 2.25 mg/kg/day.

Early publications described the phenotype of treated embryos in detail; histopathological assessments of the anomalies were made preferentially utilising near-term embryos. Comparisons between control and treated embryos at gestational stages more relevant to the period of foregut division became feasible as imaging modalities grew more advanced, and the focus of research moved towards the mechanical disturbances of development. However, extensive molecular analysis is required to discern which signalling pathways are involved in the regulation of foregut division in both normal and adriamycin-treated embryos. Ioannides et al. [2002] achieved adaption of the model to the mouse, recognising the advantage of utilising mice for molecular study. Extensive transferable knowledge from transgenic mouse lines and gene databases, as well as numerous molecular research tools, are readily available in the mouse, it being the developmental biologists' vertebrate model of choice [Bogue, 2003]. The AMM was hence established and has been reliably replicated to investigate OA/TOF and validated to display a full range of VACTERL anomalies [Dawrant et al., 2007b]. Although the direct action of adriamycin in causing these defects is unknown, the close anatomical resemblance of treated embryos to the human phenotype of VACTERL-associated OA/TOF encourages investigation of the model for embryological errors in tandem with continuing endeavours to clarify normal oesophageal development.

Adriamycin has also been shown to induce VACTERL anomalies in association with caudal regression syndrome in treated chick embryos by Naito et al. [2009] who reported distribution of drug to caudal epithelium and to the foregut. This suggests that the mechanism by which adriamycin acts could be consistent across vertebral species.

Observations of Phenotype of the Model

Adriamycin-treated pregnancies have a higher rate of embryo resorption than controls in a dose-related manner [Orford and Cass, 1999]. Treated embryos have increased amniotic fluid volume when OA is present, have lower birth weight and show significant developmental delay compared to control embryos examined at the same gestational day [Franca et al., 2004]. Variable proportions of embryos exhibiting defects have been achieved by different groups, perhaps influenced by dosing regimens, breed and animal husbandry techniques; however, a 100% rate of at least one VACTERL anomaly can be achieved [Gillick et al., 2008].

Between 28% and 93% of ARM embryos have tracheo-oesophageal malformations [Diez-Pardo et al., 1996; Merei et al., 1997b; Qi et al., 2001; Gillick et al., 2003; Xiaomei et al., 2012]. A spectrum of these anomalies have been described in the ARM, the near-term embryo most frequently (80%) displaying an upper OA pouch with distal TOF [Merei et al., 1997b]. This defect is identical to Gross classification C type OA/TOF, the most common anatomical variant seen in humans (86%) [Harmon and Coran, 1998]. The upper oesophageal blind-ending pouch was seen to arise from the dorsal wall of the distal pharynx, lined with stratified squamous epithelium, and end at the level of cricoid cartilage [Merei and Hutson, 2002]. Beasley et al. [2004] reported pouch development from the dorsal wall of the distal pharynx at E15 in the ARM, and this late outgrowth of a pouch was also described by Possoegel et al. [1998]. Pure OA without fistula, complete laryngotracheal cleft (undivided oesophago-trachea), tracheal atresia, tracheal agenesis, and stomach agenesis have also been recorded in the model [Merei et al., 1998b; Qi and Beasley, 1999a, b]. In the trachea, malformed and deficient cartilage has been described, and correlation of these findings with the high rate of tracheomalacia afflicting neonates with OA/TOF was intimated [Qi et al., 1997b; Pole et al., 2001]. The lungs of treated embryos were hypoplastic and had decreased airway branching; however, histological findings were similar to controls

[Xiaomei et al., 2012]. Other tracheo-oesophageal anomalies including foregut duplications and bronchopulmonary foregut malformations, such as an ectopic lung or bronchus arising from the oesophagus, are reported in the model, indicating these may have a similar developmental aetiology to OA/TOF in the ARM [Qi and Beasley, 1999b; Qi et al., 2001].

In CBA/Ca mice, Ioannides et al. [2002] induced tracheo-oesophageal malformations in 47% of embryos at a dose of 4 mg/kg/day of adriamycin on E6 and 7. At an increased dose of 6 mg/kg administered on E7 and 8, 50% of surviving embryos had tracheo-oesophageal anomalies when examined at E18 [Dawrant et al., 2007a]. A detailed morphological examination of the tracheo-oesophageal malformations in the AMM at a range of gestational stages in wholemount specimens was conducted by Hajduk et al. [2011b] using fluorescently labelled antibodies against specific markers expressed in the foregut (fig. 1). The spectrum of malformations perceived included: complete atresia of the entire foregut, foregut stenosis, OA with upper pouch, total laryngotracheal cleft, tracheal atresia, fistula with proximal origin at the carina or bronchus, and stomach agenesis. Interestingly, the distribution of defect type changes with gestational stage, OA with upper pouch and TOF being present in a higher proportion of embryos at later stages. A defect that appears to be a complete laryngotracheal cleft at E10 may yet undergo further morphological changes and evolve into a classical OA with distal TOF. Alternatively, a higher number of late resorptions may occur in embryos afflicted with severe defects.

The fistula is a key structure in the successful surgical reconstruction of OA. It is freed at the point of tracheal connection in the case of a distal TOF and anastomosed to the upper oesophageal pouch where feasible. Postoperatively, it is hoped the fistula would facilitate coordinated swallowing as a primary outcome. In reality, this structure may have an innate incapacity to fulfil these expectations. Disordered lower oesophageal motility and GORD frequently complicate OA/TOF in children postoperatively and cause long-term morbidity [Tomaselli et al., 2003]. These may be surgical complications or could be congenital in nature. It is vitally important to comprehend what the functional capabilities of the fistula might be in a developmental context, and to this end, debate exists as to whether it consists of oesophageal or respiratory lineage tissue.

In humans, the fistula is most commonly seen to arise from the carina (the point of tracheal bifurcation into the bronchi) and extends to the stomach. It has been shown

Fig. 1. 3D OPT images of foregut separation in wholemount embryos with HNF3 β immunolocalisation. **A** Normal embryo and **a** 3D surface representation of HNF3 β immunolocalisation in a normal embryo, both with normally developing lung buds. **B–D** AMM embryos with notochord branching (red stars) positionally associated with tracheo-oesophageal malformations (green arrows). **E** 3D surface representation of AMM embryo with a thick notochord branch and foregut atresia. fp = Floor plate; nc = notochord; lb = lung bud; fg = foregut; tr = trachea; st = stomach; pa = pancreas. Scale bars as indicated. Adapted from Hajduk et al. [2011b].

to contain cartilage as well as oesophageal musculature in its walls and to be lined by respiratory and oesophageal epithelium [Hokama et al., 1986; Yoo et al., 2010]. In the ARM, the fistula most often arises from the left main bronchus and can also originate from the posterior wall of the trachea. Crisera et al. [1999a] have reported that the fistula arises as the posterior branch of a tracheal trifurcation with the main bronchi, elongates caudally and fistulises into the stomach. Splide et al. [2002a] also reported a discontinuity between the fistula and the stomach, while other authors reported that the fistula developed from caudal foregut maintaining a connection with the stomach throughout [Merei et al., 1997b; Sasaki et al., 2001]. The fistula is lined with ciliated columnar respiratory epithelium from its proximal origin, transitioning into stratified squamous oesophageal epithelial lining at a variable distance caudally [Merei et al., 1997a]. Tracheal cartilage has been identified in the fistula wall but also have muscle layers consistent with normal lower oesophagus [Merei et al., 1997a; Cheng et al., 1999].

GORD and disordered swallow in OA/TOF may be related to developmentally derived failings of muscle or nerve tissue. In the ARM, TOF musculature has been recorded as deficient by some authors and intact by others [Cheng et al., 1999; Crisera et al., 2000b]. Aberrant course

and branching of the vagus and recurrent laryngeal nerves were noted in the model as well as reduced nerve plexus density and disordered ganglia in the TOF [Qi et al., 1997a, c]. Moreover, there were deranged levels of some neuropeptides and neural markers in the TOF suggesting atypical innervation [Qi et al., 1997a; Cheng et al., 1999]. Tugay et al. [2003b] postulated that altered gastric smooth muscle contractility and lower oesophageal reactivity in the ARM to muscle-active substances could be related to GORD pathogenesis.

Nkx2.1 is a gene expressed specifically in respiratory designated or definitive tissue, and its human homolog has been detected in resected fistula specimens [Minoo et al., 1999; Spilde et al., 2002b]. In the ARM, the fistula has been shown to be *Nkx2.1* protein positive [Crisera et al., 1999b]. In the AMM, Ioannides et al. [2002] reported that the fistula was *Nkx2.1* protein negative and expressed *Sox2*, which is usually restricted to oesophageal tissue following foregut division. Hajduk et al. [2011b] detected *Nkx2.1* gene expression in the fistula at E11 in the AMM, but not at later stages. Respiratory tissues exhibit specialised properties including branching morphogenesis [Hogan et al., 1997]. In the ARM, the fistula is not normally seen to branch in vivo, but it can be induced to branch when cultured in normal or respiratory mesen-

chyme and in response to fibroblast growth factors [Crisera et al., 2000c; Spilde et al., 2004; Xiaomei et al., 2012]. The evidence to date suggests that the fistula may arise from a bi-potential origin which can be induced under certain conditions to establish a respiratory identity when exposed to the appropriate mesenchymal signals.

The Notochord

One of the most striking and pivotal discoveries in adriamycin-treated rodent embryos was abnormality of the notochord, a transient but crucial structure in developing vertebrate embryos. Its presence defines members of the chordate phylum. The notochord is derived from the cells of the node as it regresses during gastrulation. These cells initially lie adjacent to the endoderm. They organise into a rod-shaped structure as the endoderm layer is folding to create the foregut. The notochord rod moves dorsally away from the foregut towards the neural tube in a process termed delamination, and comes to lie adjacent to the neural floor plate, eventually being re-sorbed to form part of the nucleus pulposus of the vertebral discs [Jurand, 1974]. The notochord is a potent source of signalling during organogenesis, acting as a primary organiser; it patterns cells for dorso-ventral, antero-posterior and right-left orientation [Cleaver and Krieg, 2001]. It expresses signalling molecules including Shh, which is known to induce development of foregut-derived organs such as the pancreas [Hebrok et al., 1998]. Notochord signals direct somite formation, and transplanted notochord can induce the formation of ectopic neural plate adjacent to it [Straaten et al., 1988; Bitgood and McMahon, 1995].

Abnormalities of the notochord were consistently detected in the ARM from its conception in up to 60% of embryos [Diez-Pardo et al., 1996; Merei et al., 1998a; Possoegel et al., 1999]. The structural defects encountered were given many terms, including branching, splitting, hypertrophy, bifurcation, duplication, tethering, or bending, all referring to the appearance of the notochord failing to delaminate away from the foregut at points along its length [Williams et al., 2001]. With the benefit of 3D wholemount imaging and specific immunohistochemistry to fluorescently label the notochord and foregut, Hajduk et al. [2011b] could detect notochord branches in up to 94% of treated mouse embryos, evidently being the most consistent anomaly seen in the AMM. The vicinity and thickness of these branches could be spatially and quantifiably related not only to the site and severity of

foregut abnormalities, but also to the location and extent of disturbed gene expression in foregut endoderm and mesoderm (fig. 1 and 3) [Hajduk et al., 2011b]. These notochordal abnormalities have also been identified as spatially related to the position of other intestinal atresias in the ARM [Gillick et al., 2002b].

Interestingly, notochord defects are not unique to adriamycin models. In humans, notochordal defects have been described in embryos with VACTERL anomalies including OA/TOF, although reports in the literature are sparse [Elliott et al., 1970], and split notochord syndrome describes notochord anomalies, neural tube defects and gut duplications or cysts [Bentley and Smith, 1960; Meller et al., 1989]. In the ethylenethiourea-induced model of anorectal malformations in rats, the notochord had structural abnormalities [Qi et al., 2003], and Noggin knockout transgenic mice display notochordal branches in association with tracheo-oesophageal malformations [Que et al., 2006]. The significance of these defects may not have been initially obvious; however, given the powerful patterning influence of the notochord, the obvious structural deformity seen in adriamycin-treated embryos may be echoed by signalling irregularities arising from it [Orford et al., 2001; Gillick et al., 2003; Hajduk et al., 2011b].

Other VACTERL Anomalies

Utilising the ARM, many groups have successfully generated embryos with a wide range of VACTERL malformations, and Dawrant et al. [2007b] additionally validated the AMM as a model for the association. Renal abnormalities are prominent in human VACTERL, and urogenital defects can be detected in up to 100% of ARM embryos and are also present in the AMM [van Heurn et al., 2002; Dawrant et al., 2007b; Gillick et al., 2008]. The most commonly reported defect is unilateral or bilateral hydronephroureter in association with either bladder agenesis or bladder hypoplasia [Liu et al., 1999; Liu and Hutson, 2001; Franca et al., 2004; Mortell et al., 2005]. Other anomalies include renal agenesis, uretero-urethral/urogenital fistula and urethral agenesis. Temelcos and Hutson [2004] performed microscopic examination of affected kidneys showing abnormal nephron architecture with thin medulla and dilation of collecting ducts and tubules. Anorectal malformations are present in a significant proportion of treated embryos. Imperforate anus is associated with short tails and recto-urethral and recto-vaginal fistulas have been described in males and

females, respectively [Merei, 2002], mimicking the human malformation closely [Peña and Levitt, 2006]. Cloacal malformations have also been described in the model [Liu and Hutson, 2000].

All neonates diagnosed with OA/TOF undergo screening for cardiovascular defects, as this is the most frequent anomaly occurring in association with OA/TOF and is a significant prognostic factor [Spitz et al., 1994]. Cardiovascular anomalies in the ARM include VSD, ASD and right-sided aortic arch most frequently; aberrant left innominate artery, retro-oesophageal subclavian artery and single umbilical artery have also been described [Qi et al., 1997d; Merei, 2003]. Skeletal anomalies observed in the model include asymmetry, wedging and butterflying of the vertebra, rib and sternal abnormalities, and limb deformities due to delayed ossification resulting in bowing of long bones [Kotsios et al., 1998; Xia et al., 1999; Abu-Hijleh et al., 2000]. Interestingly, single or multiple gastrointestinal atresias, including duodenal atresia, are frequently seen in this model [Fourcade et al., 2001; Merei, 2004]. Duodenal atresias have been reported occurring in association with VACTERL anomalies in humans, and some experts advocate that they should be named as part of the association [Rittler et al., 1996].

Although the ARM and AMM demonstrate the full gamut of VACTERL anomalies, the focus of research in the model has been tracheo-oesophageal malformations. Beyond the phenotypic observations summarised here, the remainder of the abnormalities arising in treated embryos have not been subject to in-depth research. However, given that a common insult has been applied, findings in relation to perturbed foregut division will have relevance to the developmental origins of all these defects, and it may be useful to examine for similar disturbances in the anlagen of these structures in this model in the future.

Disturbances of Cell Mechanisms in the Model

The early developmental errors from which tracheo-oesophageal malformations arise are not well understood. Even the normal embryological steps leading to foregut division defy consensus [Kluth and Fiegel, 2003]. Numerous theories compete to clarify these complex morphogenetic processes and, when explaining the developmental origins of OA/TOF, individual groups reflect varied interpretations of normal events. The classical theory of foregut separation describes budding of the respiratory primordia from the ventro-lateral walls of the

foregut followed by the appearance of lateral endodermal ridges that advance into the lumen of the foregut and meet in the midline, septating the foregut into 2 distinct structures [Gray and Skandalakis, 1972]. This septation is then thought to advance cranially, and elongation of the newly formed oesophagus and trachea progresses caudally or possibly bidirectionally [Cardoso and Lu, 2006]. In this chain of events, failure of septation would account for the development of tracheo-oesophageal malformations.

The existence of the lateral endodermal wall septum-forming structures is not agreed upon by all [Zaw-Tun, 1982]. O'Rahilly and Muller [1984] argue that the trachea forms by lengthening of a respiratory outgrowth from a single site in the ventral foregut (referred to as the Water and Tap theory). The designation of cells destined for respiratory lineage in ventral foregut proximal to the origin of the respiratory primordia, as indicated by the expression of *Nkx2.1* [Ioannides et al., 2010], would however suggest that ventral endoderm cranial to the level of lung budding will go on to form tracheal tissue. Ioannides et al. [2010] examined lengthening in divided and undivided foregut between fixed points in the AMM and control embryos. The normal cranial undivided foregut, measured from the pharynx to the upper limit of respiratory structures, significantly shortened between E11.5 and E12.5, suggesting that progressive septation is a key mechanism. In treated mouse embryos, there was a failure of septation with normal degree of lengthening in the persisting undivided foregut [Ioannides et al., 2010].

Programmed cell death or apoptosis as a cellular mechanism facilitating foregut division has been proposed to be disturbed in OA/TOF by Qi and Beasley [1998]. They described a temporo-spatial pattern of apoptosis in the normally dividing foregut encompassing 3 phases, initiated by proliferation of the respiratory primordia to bud from the ventro-lateral foregut wall. Apoptosis, specifically located at the lateral epithelial ridges (the laryngotracheal grooves), induced collapse of the lateral foregut walls to separate the oesophagus and trachea; however, no septum was detected. Rapid proliferation of both the oesophagus and trachea along with interposition of mesenchyme between the structures completed the process [Qi and Beasley, 2000; Williams et al., 2003]. In the ARM, lack of apoptosis at the lateral epithelial ridges was perceived to result in failure of the foregut to divide [Williams et al., 2000]. A similar change in the pattern of apoptosis at the lateral foregut wall was reported in the ARM by Orford et al. [2001] in addition to reduced cellular proliferation in paraxial and perinotochordal meso-

Fig. 2. Detection of E-cadherin during notochord delamination in control (**A, a**) and AMM (**B, b, c**) embryos using immunolocalisation and confocal microscopy. Ectopic distribution of E-cadherin (in red) in the extracellular matrix of the notochord cells in treated embryos is seen. fp = Floor plate; fg = foregut; nc = notochord. Scale bars as indicated. Adapted from Hajduk et al. [2012].

derm. Conversely, Zhou et al. [1999] found evidence of increased programmed cell death in the ARM, and Glick et al. [2002a] did not find any change in the pattern or rate of apoptosis between treated and control embryos. Ioannides et al. [2010] also reported a change in the pattern of apoptosis in each of the AMM, *Shh* and *Nkx2.1* knockout mouse transgenic lines, all of which exhibit tracheo-oesophageal malformations. The group additionally examined the *Apaf1* mutant mouse in which apoptosis is inhibited and demonstrated foregut division proceeding in an orderly fashion [Ioannides et al., 2010]. Therefore, it seems that although apoptosis occurs in these rapidly changing tissues and may exhibit altered patterns in adriamycin models, it is unlikely that programmed cell death is the primary mechanism by which normal foregut separation is achieved.

Cell-to-cell interactions are fundamental to cell behaviour. Notochord cells may reprogram the identity of the foregut endoderm by prolonged direct cellular contact. This extended interaction is conceivably generated by defective intracellular adhesion. Proteins implicated

in intracellular adhesion such as fibronectin have heightened expression in anomalous notochord branches in the ARM [Mortell et al., 2004b]. Li et al. [2007] proposed that prolonged cellular adhesion permitted the notochord to appropriate dorsal foregut cells, inducing atresia in the prospective oesophagus by decreasing cell numbers. Expression of intracellular adhesion proteins pan-cadherin and N-CAM were upregulated in the proximal atretic oesophagus, fistula and the trachea of the ARM, indicating that irregular cell adhesion behaviour is not confined to the notochord in adriamycin treated embryos [Tugay et al., 2003a].

Cell morphology and shape also influence cell behaviour. It is conceivable that disrupted intracellular adhesion could influence cell behaviour not only by abnormally prolonging cell-to-cell interaction and signalling, but also by directly altering cell shape and polarity. The establishment of cell polarity, the sorting of molecules into cellular domains, in epithelial cells is a crucial step in enabling cell function [Tepass, 2012]. Cadherins have a vital role in determining cell migratory behaviour and tissue differentiation [Kikuchi et al., 2009] and are involved in stabilising cell polarity [Grindstaff et al., 1998; Tepass et al., 2000]. In the AMM, E-cadherin expression, normally specific to epithelial cells, was detected in the extracellular matrix of cells in aberrant notochord branches using confocal microscopy (fig. 2) [Hajduk et al., 2012]. Interestingly, in human breast cancer cells, adriamycin encouraged intracellular adhesion via upregulation of E-cadherin [Yang et al., 1999]; therefore, excess E-cadherin could alter cell behaviour by inducing intracellular adhesion as well as influencing cell polarity.

Laminins are ubiquitously present in cell basement membrane [Hamill et al., 2009]. In the AMM, the notochord was hindered from establishing a basal lamina independent of the foregut and abnormal streaks of laminin connected the structures during disrupted delamination [Hajduk et al., 2012]. Arrangement of notochord cells in a rigid structure is thought to facilitate elongation [Adams et al., 1990]. Failure to synthesise an intact basal membrane may affect turgor within the notochord rod, impair delamination and induce structural defects. It has been suggested that failure of the notochord to delaminate in adriamycin-treated embryos may affect the foregut structure mechanically by impingement or by traction [Qi and Beasley, 1999c; Merei, 2004]. Traction, generated by excessive adhesion, could be propagated by differential growth rates in adjacent tissues inducing structural deformity in both foregut and notochord [Sutcliffe and Hutchins, 1994].

Defects in mesodermal organisation surrounding the foregut endoderm could result in tracheo-oesophageal malformations. The mesoderm maintains many functions including reciprocal signalling with adjacent endoderm and the establishment of fields into which epithelial organs can proliferate, and subsequently forms specialised tissue mesenchyme. Epithelial mesenchymal interactions are known to be crucial to organogenesis in the lung and many other tissues [Thesleff et al., 1995; Hogan and Yingling, 1998]. In the ventral foregut mesoderm, condensed cellular patterns are associated with the outgrowth of the lung buds [Morrisey and Hogan, 2010]. Possoegel et al. [1998] and others have reported disturbances in mesodermal cell pattern, describing the cellular architecture as disorganised and loosely arranged around the foregut in the ARM with loss of the circular pattern detected in control embryos at both E12 and E13 [Sasaki et al., 2001]. As well as signalling derangements discussed anon, failure of these fields to form could physically deter organogenesis; however, the detail of this mechanism is unexplained.

Mechanistic disturbances and molecular signalling derangements are not independent processes, but are inextricably linked. Signals received by the cell dictate function and morphology [Tam et al., 2003]. Conversely, the shape of a cell and the organisation within it influence how it responds to further stimuli. The abnormalities of cellular behaviour and morphology within the notochord branches are positionally associated with zones of abnormal gene expression in the adjacent foregut and furthermore correlate with the site of tracheo-oesophageal malformations [Hajduk et al., 2011b].

Molecular Patterning

Morphogenetic changes during development are influenced by complex interacting signalling pathways. Positional information, including instructions for differentiation, proliferation, migration, and many other aspects of cell function are derived from these signals. The data available for the molecular control of organogenesis of the oesophagus is far from comprehensive. The opportunity for this type of investigation is limited in the rat as species-specific resources such as molecular probes or extensive genetic databases are not readily available. The adaption of the model to the mouse, which in contrast offers these advantages, highly benefitted research in this field.

Preceding lung bud formation, signals to the foregut endoderm cells designate specific organ domains. This is elegantly shown by the reciprocal gene expression of *Nkx2.1* ventrally and *Sox2* dorsally in the foregut, demarcating the future respiratory and oesophageal tissue domains, respectively [Que et al., 2007]. Knockout transgenic mice for both of these genes have tracheo-oesophageal malformations [Minoo et al., 1999; Que et al., 2007]. In humans, *SOX2* gene mutation causes anophthalmia-oesophageal-genital syndrome in which OA/TOF is present [Williamson et al., 2006]. In virtual sections of 3D reconstructed AMM embryos, Hajduk et al. [2011b] found dorsal *Sox2* expression to be lost at a specific level in the foregut adjacent to notochord branches. It is apparent that dorso-ventral (d-v) patterning of the foregut cells by signalling pathways is essential in regulating organogenesis. Similarly, many other genes exhibit specific d-v patterns in the foregut endoderm and mesoderm and some of these will be discussed below as they relate to the ARM and AMM. The upstream modulators that establish cell identity in the foregut endoderm are likely many and complex.

Shh Pathway

The *Shh* signalling pathway is a prime candidate for disturbance in OA/TOF. *Shh* is fundamental to developmental processes in vertebrates and invertebrates [Hooper and Scott, 2005]. It functions in antero-posterior, dorso-ventral and right-left patterning in the embryo [Johnson et al., 1994; Hamada et al., 2002]. It is expressed in both the notochord and foregut endoderm, and disturbances in expression are known to affect the development of foregut-derived organs such as the pancreas [Kim et al., 1997; Litingtung et al., 1998]. Transducers of the *Shh* pathway include receptors *Smoothed* and *Patched* (*Ptc*), signalling complex proteins *Gli2* and *3* and effector gene *Foxf1*, which is expressed in ventral foregut mesoderm. Transgenic mouse lines for *Shh*, *Gli2* and *3* and *Foxf1* all have tracheo-oesophageal malformations [Litingtung et al., 1998; Motoyama et al., 1998; Mahlapuu et al., 2001]. In humans, *SHH* mutations have been identified in patients with OA/TOF, anorectal malformation and holoprosencephaly [Cohen, 1989], *GLI3* mutation causes Pallister Hall Syndrome with OA/TOF phenotype [Johnston et al., 2005], and mutations or deletions affecting *FOXF1* and the *FOXF1* microdeletion cluster are associated with VACTERL-like phenotypes [Stankiewicz et al., 2009; Shaw-Smith, 2010].

Arsic et al. [2003] reported a peak of *Shh* expression at earlier embryonic days in the normal rat foregut which

Fig. 3. OPT image of *Shh* gene expression in an adriamycin-treated embryo. A thick notochord branch is associated with loss of normal ventral restriction of *Shh* expression (yellow) in the foregut. In section **a** the normal pattern is seen with dorsal foregut outlined in red. In sections **b** and **c** dorsal shift of *Shh* expression is seen adjacent to the location of the notochord branch. fp = Floor plate, nc = notochord, fg = foregut. Adapted from Hajduk et al. [2011b].

declines towards term. ARM embryos lost this early peak of expression, and instead, there was a persistent low level of *Shh* gene and protein expression in the foregut. Additionally, a d-v pattern of *Shh* expression in foregut en-

doderm, described as ventrally restricted to the prospective trachea, was lost in the ARM [Arsic et al., 2004]. Notochord branches have been confirmed to express *Shh* [Gillick et al., 2003]. *Shh* promotes its own expression in target tissues and is known to act in a concentration gradient-dependant manner [Bumcrot and McMahon, 1996; Zeng et al., 2001]. In the neural crest, there was reduced auto-induction of *Shh* expression when the notochord was ectopically positioned closer [Goh et al., 1997]. The concentration of *Shh* received by the foregut in the ARM could arguably be higher due to either increased volume of notochord tissue or the proximity of the notochord branch to the foregut, and in response, the endodermal expression of *Shh* may decline [Mortell et al., 2004a]. *Gli2* mRNA levels were lower in the fistula than in the oesophagus in the ARM, and the fistula, although *Shh* negative, was induced to branch by exogenous *Shh* in culture [Spilde et al., 2003a, c].

Ioannides et al. [2003] detected a specific d-v pattern of *Shh* expression in the foregut of normal mice. *Shh* was expressed only in the ventral endoderm prior to foregut division, a shift then occurring from ventral-only to dorsal-only expression at the site of immediately impending separation. *Shh* expression persisted in the oesophagus and was absent in respiratory tissues once separation had concluded. This shift in expression of *Shh* was lost in the foregut endoderm of AMM embryos with tracheo-oesophageal malformations, where *Shh* was diffusely present with no identifiable d-v restriction. *Ptc* expression was unchanged between treated embryos and controls [Ioannides et al., 2003]. With optical projection tomography (OPT), it was possible to visualise *Shh* and *Foxf1* expression patterns in the foregut in detail [Sato et al. 2008]. Hajduk et al. [2011b] confirmed the diffuse expression pattern of *Shh* in the AMM foregut and additionally found an abnormal site of *Shh* expression in the dorsal foregut which correlated with the vicinity and thickness of notochord branches (fig. 3). *Foxf1* expression pattern in the mesoderm was also altered, with a loss of ventral restriction and a more diffuse expression pattern around the AMM foregut associated with the position of the notochord branches [Hajduk et al., 2011b]. *Gli1*, 2 and 3 expression were unchanged in AMM [Hajduk et al., unpubl. results].

Wnt Pathway

Wnt signalling plays a major role in organising the vertebrate body plan [Logan and Nusse, 2004]. It has been shown to be essential to sustain notochord progenitor cell fate and for extension of the notochord during embryo-

genesis [Ukita et al., 2009]. Defined roles in foregut division have not been ascertained for Wnt ligands, some of which are expressed in the foregut mesoderm (Wnt2 and 2b) and endoderm (Wnt7b) [Rajagopal et al., 2008; Goss et al., 2009]. Wnt pathway inactivation inhibits foregut separation [Harris-Johnson et al., 2009]. *Wnt2/2b* knockout mice display complete lung agenesis without foregut separation and altered d-v patterning of the foregut with absence of Nkx2.1 expression in ventral endoderm [Goss et al., 2009]. Wnt7b inactivation results in lung hypoplasia [Shu et al., 2002]. Miller et al. [2012] found that cross-talk of Wnt2 mesodermal and 7b endodermal signalling from complimentary expression domains was crucial in anterior foregut organogenesis both in vivo and in vitro. In the AMM, however, Wnt2 expression was not found to be different in the mesoderm of the foregut versus controls, although it was detected in the mesoderm around the fistula, and nor was Wnt7b expression altered [Hajduk et al., 2011a].

BMP Pathway

The role of BMP pathway signalling in foregut division is uncertain; however, there are indications that elements of this pathway could be deranged in OA/TOF. BMP ligands are expressed in foregut endoderm and mesoderm and in the notochord [McMahon et al., 1998; Weaver et al., 2000; Li et al., 2008]. BMP receptors in the ventral foregut may act via Sox2 inhibition to permit tracheogenesis [Domyan et al., 2011]. *Bmp4* knockout mice have tracheal agenesis and transgenic knockout mice for *Noggin*, a BMP pathway antagonist, have tracheo-oesophageal malformations in association with notochord branches, a phenotype which was rescued by ablating *Bmp7* or by reducing the gene dose of *Bmp4* by 50% [Que et al., 2006; Li et al., 2007]. It seems that tightly regulated BMP signalling is implicit in normal foregut separation; however, in the AMM, *Bmp4* expression in the ventral foregut mesoderm was not altered [Hajduk et al., unpubl. results].

Retinoic Acid Signalling

RA is the active metabolite of vitamin A. Offspring of rats reared on a vitamin A-deficient diet exhibit severe congenital malformations including lung agenesis and an undivided foregut [Wilson et al., 1953; Dickman et al., 1997]. Various retinoic acid nuclear receptors (RARs) transduce retinoid signals, and the double mutant knockout mice for *RAR α* and *RAR β* have absent oesophago-tracheal septum [Mendelsohn et al., 1994]. When cultured with RAR antagonist, lung budding failed in fore-

gut explants [Mollard et al., 2000]. Deletion of retinaldehyde dehydrogenase 2, an enzyme essential for RA synthesis in mouse embryos, also leads to absence of lung buds [Desai et al., 2006]. Nkx2.1 expression was significantly reduced in the ventral foregut endoderm in embryos with deficient RA signalling (Desai et al., 2004). In the AMM, however, *RAR α* and *RAR* expression were not different to controls [Hajduk et al., unpubl. results].

FGF Signalling

FGF signalling is synonymous with the regulation of lung organogenesis [Warburton and Bellusci, 2004]; however, its role in foregut separation is undefined. In the ARM, FGF signalling was investigated for influence on the fistula in the context of this structure deriving from respiratory lineage. The fistula was positive for *Fgf1* and *Fgf10*, but not *Fgf7* mRNA. FGF receptor *Fgf2rIIIb*, to which *Fgf7* and 10 obligately bind, was shown to be absent or downregulated in the fistula compared to respiratory structures [Crisera et al., 2000c; Spilde et al., 2003b, 2004]. These findings implicated FGFs as possible determinants of branching ability in the respective tissues. ARM fistula epithelium in recombinant culture displayed branching in response to *Fgf1* [Crisera et al., 2000a]. In normal mice, *Fgf10* gene expression was detected in mesenchyme at the tips of the lung buds, and the onset of *Fgf10* expression was shown to be delayed in AMM embryos [Hajduk et al., 2010b].

Other Pathways

T-box transcription factor (*Tbx4*) is coexpressed with *Fgf10* in ventral foregut mesoderm [Gibson-Brown et al., 1998]. In chicks, Sakiyama et al. [2003] reported ectopic mesodermal induction of *Tbx4* and *Fgf10* resulting in ectopic expression of Nkx2.1 in endoderm and subsequent ectopic lung budding. Failure of foregut division was seen in these embryos and ectopic *Tbx4* or *Fgf10* could each induce the expression of the other in the mesoderm, indicating a feedback loop exists between these genes. However, in mice, inactivation of the *Tbx4* gene did not prevent lung bud formation [Naiche and Papaioannou, 2003], and in the AMM, *Tbx4* expression was unchanged between treated and control embryos [Hajduk et al., 2010a].

Hox genes are a large family of major patterning genes which confer axial positional information in the vertebrate and are best described in the development of the axial skeleton [Mallo et al., 2010]. *Hox* subgroups have been shown to be expressed in the foregut mesoderm and endoderm [Bogue et al., 1996; Pitera et al., 1999]. Various

Hox gene mutations induce tracheal or oesophageal malformations in transgenic mice [Boulet and Capecchi, 1996; Aubin et al., 1997; Szumska et al., 2008]. Calonge et al. [2007] recorded reduced protein and RNA levels of *Hoxa3*, *Hoxb3*, *Hoxd3*, and *Hoxc4* in AMM lungs.

These pathways do not act independently of each other but instead cooperate in a complex regulatory circuit. *Shh* stimulates *Wnt2* and *Bmp4*, restricts *Fgf10* as well as upregulating *Noggin* expression in the foregut mesoderm [Pepicelli et al., 1998; Lebeche et al., 1999; Weaver et al., 2003]. *Bmp4* and *Fgf10* have opposing roles in the control of lung budding and *Fgf10* activates *Bmp4* signalling [Bellusci et al., 1997; Weaver et al., 2000]. *Bmp4* downregulates *Foxf1* expression [Mahlpuu et al., 2001]. *Wnt7b* signalling promotes the expression of *Bmp4* and *Fgfr2* in the epithelium [Shu et al., 2002; Rajagopal et al., 2008], and *Fgf10* is RA signalling responsive in the foregut mesoderm [Desai et al., 2006; Chen et al., 2007]. This is by no means an exhaustive account of the types of signalling interrelationships recognised in foregut development and additionally does not examine the transitional nature of these signals with regard to timing and location, or the variety of influences they may exert on different target tissue types.

It is evident that temporo-spatial d-v patterning of the foregut is a pivotal governing apparatus of foregut separation. Well demarcated zones of disturbed expression of *Shh*, *Foxf1* and *Sox2* have been identified in the foregut of the AMM. These disturbances have been shown to be not only spatially related to the locale of notochord branching and tracheo-oesophageal malformations, but the extent of the deranged expression patterns are quantifiably associated with the severity of the foregut malformation and thickness of the notochord branch (fig. 3) [Hajduk et al., 2011b].

Imaging Techniques

Interpretations of complex and rapidly changing morphological events with serial histological sections have been frequently cited by authors as limited. While these analyses have provided valuable insight into the spectrum of malformations of OA/TOF, they are restricted to partial viewing of the effects, lack of visualisation of the whole embryo impeding overall understanding of the morphology. Studies have been additionally constrained in the examination of gene expression patterns to sections or external whole embryo patterns. A powerful tool for 3D imaging of mouse embryos has emerged, namely

OPT. This is a rapid technique for 3D imaging of whole biological tissue specimens, recording morphology while also allowing visualisation of the total tissue distribution of RNA, protein or histological stains in developing organs using virtual sections of 3D reconstructed data as demonstrated in figures 1 and 3 [Sharpe et al., 2002; Alantalo et al., 2007; Summerhurst et al., 2008]. At a cellular level, 3D imaging is also feasible using confocal laser microscopy for fluorescently labelled markers allowing close examination of intracellular interactions (fig 2).

Integrating Morphological and Molecular Aspects of the Model

Often, when formulating embryological explanations for congenital conditions such as OA/TOF, correlations of structural defects and signalling disturbances are not observed. However, morphological and molecular elements are inextricably connected in ordered cell function and in embryogenesis. Molecular control determines physical cell characteristics such as shape and polarity, and these factors influence how the cell responds to further stimuli, whether chemical or physical, determining the ultimate morphological outcome. The intricacies of cell function, organogenesis and the embryo as a whole demand integrated descriptions of these multifactorial developmental errors. The types of research questions posed should align with this mandate and will lead to more meaningful results in an embryological context.

An excellent example of this is the recognition of close spatial relationship between the vicinity of notochord branches, the position of tracheo-oesophageal malformations and the zone of disturbed gene expression in the AMM. Embracing new imaging techniques, such as OPT and confocal microscopy, has enabled the integrated examination of morphological and molecular disturbances in the model from whole embryos down to a cellular level. Tissue structural defects do not occur in isolation in the AMM, but are temporo-spatially associated with irregularities of both cell morphology and behaviour such as alterations in intracellular adhesion and gene expression. Additionally, the volume of the tissue defect in the notochord can be quantifiably correlated with the severity of the malformation in the foregut and the extent of gene expression disturbance in both the endoderm and mesoderm.

Conclusion

The ARM, conceived by paediatric surgeons to enhance comprehension of OA/TOF pathogenesis, is associated with prolific research yield. Adaption of the model to the mouse has promoted relevance to the field of developmental biology and has facilitated interdisciplinary collaboration towards this common aim, allowing complementary work between the AMM and transgenic mouse lines to further understanding of key developmental disturbances. The AMM and ARM are the most

clinically relatable modes for OA/TOF in terms of phenotypic similarity. Distinct morphological features, irregularities of cellular mechanisms and molecular disturbances possibly implicated in OA/TOF pathogenesis have been identified to inform future work. There is now the challenge and opportunity to integrate knowledge at the molecular and morphological levels. Normal mechanisms of foregut development in correlation with the complex signalling control of foregut division continue to need definition. Adriamycin rodent models of OA/TOF and VACTERL remain applicable to these goals.

References

- Abu-Hijleh G, Qi BQ, Williams AK, Beasley SW: Development of the bones and synovial joints in the rat model of the VATER association. *J Orthop Sci* 5:390–396 (2000).
- Adams DS, Keller R, Koehl MA: The mechanics of notochord elongation, straightening and stiffening in the embryo of *Xenopus laevis*. *Development* 110:115–130 (1990).
- Alanentalo T, Asayesh A, Morrison H, Lorén CE, Holmberg D, et al: Tomographic molecular imaging and 3D quantification within adult mouse organs. *Nat Methods* 4:31–33 (2007).
- Arsic D, Keenan J, Quan QB, Beasley S: Differences in the levels of Sonic hedgehog protein during early foregut development caused by exposure to Adriamycin give clues to the role of the *Shh* gene in oesophageal atresia. *Pediatr Surg Int* 19:463–466 (2003).
- Arsic D, Cameron V, Ellmers L, Quan QB, Keenan J, et al: Adriamycin disruption of the Shh-Gli pathway is associated with abnormalities of foregut development. *J Pediatr Surg* 39:1747–1753 (2004).
- Aubin J, Lemieux M, Tremblay M, Bérard J, Jean-notte L: Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. *Dev Biol* 192:432–445 (1997).
- Beasley SW, Williams AK, Qi BQ, Vlesch Dubois VN: The development of the proximal oesophageal pouch in the adriamycin rat model of oesophageal atresia with tracheo-oesophageal fistula. *Pediatr Surg Int* 20:548–550 (2004).
- Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL: Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* 124:4867–4878 (1997).
- Bentley JF, Smith JR: Developmental posterior enteric remnants and spinal malformations: the split notochord syndrome. *Arch Dis Child* 35:76–86 (1960).
- Bitgood MJ, McMahon AP: *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol* 172:126–138 (1995).
- Bogue CW: Genetic models in applied physiology. Functional genomics in the mouse: powerful techniques for unraveling the basis of human development and disease. *J Appl Physiol* 94:2502–2509 (2003).
- Bogue CW, Lou LJ, Vasavada H, Wilson CM, Jacobs HC: Expression of *Hoxb* genes in the developing mouse foregut and lung. *Am J Respir Cell Mol Biol* 15:163–171 (1996).
- Botto LD, Khoury MJ, Mastroiacovo P, Castilla EE, Moore CA, et al: The spectrum of congenital anomalies of the VATER association: an international study. *Am J Med Genet* 71:8–15 (1997).
- Boulet AM, Capecchi MR: Targeted disruption of *hoxc-4* causes esophageal defects and vertebral transformations. *Dev Biol* 177:232–249 (1996).
- Brunner HG, van Bokhoven H: Genetic players in esophageal atresia and tracheo-oesophageal fistula. *Curr Opin Genet Dev* 15:341–347 (2005).
- Bumcrot DA, McMahon AP: Sonic hedgehog: making the gradient. *Chem Biol* 3:13–16 (1996).
- Calonge WM, Martinez L, Lacadena J, Fernandez-Dumont V, Matesanz R, Tovar JA: Expression of homeotic genes *Hoxa3*, *Hoxb3*, *Hoxd3* and *Hoxc4* is decreased in the lungs but not in the hearts of adriamycin-exposed mice. *Pediatr Surg Int* 23:419–424 (2007).
- Cardoso WV, Lu J: Regulation of early lung morphogenesis: questions, facts and controversies. *Development* 133:1611–1624 (2006).
- Chen F, Desai TJ, Qian J, Niederreither K, Lü J, Cardoso WV: Inhibition of Tgf beta signaling by endogenous retinoic acid is essential for primary lung bud induction. *Development* 134:2969–2979 (2007).
- Cheng W, Bishop AE, Spitz L, Polak JM: Abnormal enteric nerve morphology in atretic esophagus of fetal rats with adriamycin-induced esophageal atresia. *Pediatr Surg Int* 15:8–10 (1999).
- Cleaver O, Krieg PA: Notochord patterning of the endoderm. *Dev Biol* 234:1–12 (2001).
- Cohen MM Jr: Perspectives on holoprosencephaly: Part I. Epidemiology, genetics, and syndromology. *Teratology* 40:211–235 (1989).
- Crisera CA, Connelly PR, Marmureanu AR, Colen KL, Rose MI, et al: Esophageal atresia with tracheo-oesophageal fistula: suggested mechanism in faulty organogenesis. *J Pediatr Surg* 34:204–208 (1999a).
- Crisera CA, Connelly PR, Marmureanu AR, Li M, Rose MI, et al: TTF-1 and HNF-3beta in the developing tracheo-oesophageal fistula: further evidence for the respiratory origin of the 'distal esophagus'. *J Pediatr Surg* 34:1322–1326 (1999b).
- Crisera CA, Grau JB, Maldonado TS, Kadison AS, Longaker MT, Gittes GK: Defective epithelial-mesenchymal interactions dictate the organogenesis of tracheo-oesophageal fistula. *Pediatr Surg Int* 16:256–261 (2000a).
- Crisera CA, Maldonado TS, Kadison AS, Li M, Longaker MT, Gittes GK: Patterning of the 'distal esophagus' in esophageal atresia with tracheo-oesophageal fistula: is thyroid transcription factor 1 a player? *J Surg Res* 92:245–249 (2000b).
- Crisera CA, Maldonado TS, Longaker MT, Gittes GK: Defective fibroblast growth factor signaling allows for nonbranching growth of the respiratory-derived fistula tract in esophageal atresia with tracheo-oesophageal fistula. *J Pediatr Surg* 35:1421–1425 (2000c).
- Cullinane C, Cutts SM, van Rosmalen A, Phillips DR: Formation of adriamycin-DNA adducts in vitro. *Nucleic Acids Res* 22:2296–2303 (1994).
- Cullinane C, Cutts SM, Panousis C, Phillips DR: Interstrand cross-linking by adriamycin in nuclear and mitochondrial DNA of MCF-7 cells. *Nucleic Acids Res* 28:1019–1025 (2000).
- Dawrant MJ, Giles S, Bannigan J, Puri P: Adriamycin mouse model: a variable but reproducible model of tracheo-oesophageal malformations. *Pediatr Surg Int* 23:469–472 (2007a).

- Dawrant MJ, Giles S, Bannigan J, Puri P: Adriamycin produces a reproducible teratogenic model of vertebral, anal, cardiovascular, tracheal, esophageal, renal, and limb anomalies in the mouse. *J Pediatr Surg* 42:1652–1658 (2007b).
- Depaeppe A, Dolk H, Lechat MF: The epidemiology of tracheo-oesophageal fistula and oesophageal atresia in Europe. EUROCAT working group. *Arch Dis Child* 68:743–748 (1993).
- Desai TJ, Malpel S, Flentke GR, Smith SM, Cardoso WV: Retinoic acid selectively regulates *Fgf10* expression and maintains cell identity in the prospective lung field of the developing foregut. *Dev Biol* 273:402–415 (2004).
- Desai TJ, Chen F, Lü J, Qian J, Niederreither K, et al: Distinct roles for retinoic acid receptors alpha and beta in early lung morphogenesis. *Dev Biol* 291:12–24 (2006).
- Dickman ED, Thaller C, Smith SM: Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* 124:3111–3121 (1997).
- Diez-Pardo JA, Baoquan Q, Navarro C, Tovar JA: A new rodent experimental model of esophageal atresia and tracheoesophageal fistula: preliminary report. *J Pediatr Surg* 31:498–502 (1996).
- Domyan ET, Ferretti E, Throckmorton K, Mishina Y, Nicolis SK, Sun X: Signaling through BMP receptors promotes respiratory identity in the foregut via repression of *Sox2*. *Development* 138:971–981 (2011).
- Dunkern TR, Mueller-Klieser W: Quantification of apoptosis induction by doxorubicin in three types of human mammary carcinoma spheroids. *Anticancer Res* 19:3141–3146 (1999).
- Elliott GB, Tredwell SJ, Elliott KA: The notochord as an abnormal organizer in production of congenital intestinal defect. *Am J Roentgenol Radium Ther Nucl Med* 110:628–634 (1970).
- Felix JF, de Jong EM, Torfs CP, de Klein A, Rotter RJ, Tibboel D: Genetic and environmental factors in the etiology of esophageal atresia and/or tracheoesophageal fistula: an overview of the current concepts. *Birth Defects Res A Clin Mol Teratol* 85:747–754 (2009).
- Fogo AB: Animal models of FSGS: lessons for pathogenesis and treatment. *Semin Nephrol* 23:161–171 (2003).
- Fourcade L, Shima H, Miyazaki E, Puri P: Multiple gastrointestinal atresias result from disturbed morphogenesis. *Pediatr Surg Int* 17:361–364 (2001).
- Franca WM, Goncalves A, Moraes SG, Pereira LA, Sbragia L: Esophageal atresia and other visceral anomalies in a modified adriamycin rat model and their correlations with amniotic fluid volume variations. *Pediatr Surg Int* 20:602–608 (2004).
- Gianni L, Herman EH, Lipshultz SE, Minotti G, Sarvazyan N, Sawyer DB: Anthracycline cardiotoxicity: from bench to bedside. *J Clin Oncol* 26:3777–3784 (2008).
- Gibson-Brown JJ, Agulnik S, Silver LM, Papaioannou VE: Expression of T-box genes *Tbx2-Tbx5* during chick organogenesis. *Mech Dev* 74:165–169 (1998).
- Gillick J, Giles S, Bannigan J, Puri P: Cell death in the early adriamycin rat model. *Pediatr Surg Int* 18:576–580 (2002a).
- Gillick J, Giles S, Bannigan S, Puri P: Midgut atresias result from abnormal development of the notochord in an Adriamycin rat model. *J Pediatr Surg* 37:719–722 (2002b).
- Gillick J, Mooney E, Giles S, Bannigan J, Puri P: Notochord anomalies in the adriamycin rat model: a morphologic and molecular basis for the VACTERL association. *J Pediatr Surg* 38:469–473 (2003).
- Gillick J, Mortell A, Dawrant M, Giles S, Bannigan J, Puri P: The Adriamycin rat/mouse model and its importance to the paediatric surgeon. *Pediatr Surg Int* 24:113–118 (2008).
- Goh KL, Yang JT, Hynes RO: Mesodermal defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. *Development* 124:4309–4319 (1997).
- Goss AM, Tian Y, Tsukiyama T, Cohen ED, Zhou D, et al: *Wnt2/2b* and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell* 17:290–298 (2009).
- Grapin-Botton A, Constam D: Evolution of the mechanisms and molecular control of endoderm formation. *Mech Dev* 124:253–278 (2007).
- Gray SW, Skandalakis JE: Embryology for Surgeons: The Embryological Basis for the Treatment of Congenital Defects, pp 63–100 (WB Saunders, Philadelphia 1972).
- Grindstaff KK, Bacallao RL, Nelson WJ: Apiconuclear organization of microtubules does not specify protein delivery from the trans-Golgi network to different membrane domains in polarized epithelial cells. *Mol Biol Cell* 9:685–699 (1998).
- Gutteridge JM, Halliwell B: Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann NY Acad Sci* 899:136–147 (2000).
- Hajduk P, Murphy P, Puri P: Mesenchymal expression of *Tbx4* gene is not altered in adriamycin mouse model. *Pediatr Surg Int* 26:407–411 (2010a).
- Hajduk P, Murphy P, Puri P: *Fgf10* gene expression is delayed in the embryonic lung mesenchyme in the adriamycin mouse model. *Pediatr Surg Int* 26:23–27 (2010b).
- Hajduk P, Murphy P, Puri P: A role for *Wnt2* gene in the pathogenesis of tracheoesophageal fistula in the adriamycin mouse model. Presented at The Young Investigator Award Session, 12th European Congress of Paediatric Surgery, Barcelona, 2011(a).
- Hajduk P, Sato H, Puri P, Murphy P: Abnormal notochord branching is associated with foregut malformations in the adriamycin treated mouse model. *PLoS One* 6:e27635 (2011b).
- Hajduk P, May A, Puri P, Murphy P: The effect of adriamycin exposure on the notochord of mouse embryos. *Birth Defects Res B Dev Reprod Toxicol* 95:175–183 (2012).
- Hamada H, Meno C, Watanabe D, Saijoh Y: Establishment of vertebrate left-right asymmetry. *Nat Rev Genet* 3:103–113 (2002).
- Hamill KJ, Kligys K, Hopkinson SB, Jones JC: Laminin deposition in the extracellular matrix: a complex picture emerges. *J Cell Sci* 122:4409–4417 (2009).
- Harmon CM, Coran AG: Congenital anomalies of the esophagus, in O' Neill JA Jr, Rowe MI, Grosfield JL, Fonkalsrud EW, Coran AG (eds): *Pediatric Surgery*, pp 941–967 (Mosby, St. Louis, 1998).
- Harris-Johnson KS, Domyan ET, Vezina CM, Sun X: Beta-catenin promotes respiratory progenitor identity in mouse foregut. *Proc Natl Acad Sci USA* 106:16287–16292 (2009).
- Hebrok M, Kim SK, Melton DA: Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev* 12:1705–1713 (1998).
- Hogan BL, Yingling JM: Epithelial/mesenchymal interactions and branching morphogenesis of the lung. *Curr Opin Genet Dev* 8:481–486 (1998).
- Hogan BL, Grindley J, Bellusci S, Dunn NR, Emoto H, Itoh N: Branching morphogenesis of the lung: new models for a classical problem. *Cold Spring Harb Symp Quant Biol* 62:249–256 (1997).
- Hokama A, Myers NA, Kent M, Campbell PE, Chow CW: Esophageal atresia with tracheoesophageal fistula. *Pediatr Surg Int* 1:117–121 (1986).
- Hooper JE, Scott MP: Communicating with Hedgehogs. *Nat Rev Mol Cell Biol* 6:306–317 (2005).
- Ioannides AS, Chaudhry B, Henderson DJ, Spitz L, Copp AJ: Dorsoventral patterning in oesophageal atresia with tracheo-oesophageal fistula: evidence from a new mouse model. *J Pediatr Surg* 37:185–191 (2002).
- Ioannides AS, Henderson DJ, Spitz L, Copp AJ: Role of Sonic hedgehog in the development of the trachea and oesophagus. *J Pediatr Surg* 38:29–36 (2003).
- Ioannides AS, Massa V, Ferraro E, Cecconi F, Spitz L, et al: Foregut separation and tracheo-oesophageal malformations: the role of tracheal outgrowth, dorso-ventral patterning and programmed cell death. *Dev Biol* 337:351–362 (2010).
- Jacobs JJ, Ku WY, Que J: Genetic and cellular mechanisms regulating anterior foregut and esophageal development. *Dev Biol* 369:54–64 (2012).

- Johnson RL, Riddle RD, Laufer E, Tabin C: Sonic hedgehog: a key mediator of anterior-posterior patterning of the limb and dorso-ventral patterning of axial embryonic structures. *Biochem Soc Trans* 22:569–574 (1994).
- Johnston JJ, Olivos-Glander I, Killoran C, Elson E, Turner JT, et al: Molecular and clinical analyses of Greig cephalopolysyndactyly and Pallister-Hall syndromes: robust phenotype prediction from the type and position of *GLI3* mutations. *Am J Hum Genet* 76:609–622 (2005).
- Jurand A: Some aspects of the development of the notochord in mouse embryos. *J Embryol Exp Morphol* 32:1–33 (1974).
- Kikuchi M, Yatabe M, Fujiwara K, Horiguchi K, Kusumoto K, et al: Spatio-temporal relation between cadherin switching and cytogenesis of hormone-producing cells in the developing rat adenohypophysis. *Anat Sci Int* 84:155–160 (2009).
- Kim SK, Hebrok M, Melton DA: Notochord to endoderm signaling is required for pancreas development. *Development* 124:4243–4252 (1997).
- Kluth D, Fiegel H: The embryology of the foregut. *Semin Pediatr Surg* 12:3–9 (2003).
- Kotsios C, Merei J, Hutson JM, Graham HK: Skeletal anomalies in the adriamycin-exposed prenatal rat: a model for VATER association. *J Orthop Res* 16:50–53 (1998).
- Kovesi T, Rubin S: Long-term complications of congenital esophageal atresia and/or tracheoesophageal fistula. *Chest* 126:915–925 (2004).
- Lebeche D, Malpel S, Cardoso WV: Fibroblast growth factor interactions in the developing lung. *Mech Dev* 86:125–136 (1999).
- Li Y, Litington Y, Ten Dijke P, Chiang C: Aberrant Bmp signaling and notochord delamination in the pathogenesis of esophageal atresia. *Dev Dyn* 236:746–754 (2007).
- Li Y, Gordon J, Manley NR, Litington Y, Chiang C: Bmp4 is required for tracheal formation: a novel mouse model for tracheal agenesis. *Dev Biol* 322:145–155 (2008).
- Litington Y, Lei L, Westphal H, Chiang C: Sonic hedgehog is essential to foregut development. *Nat Genet* 20:58–61 (1998).
- Liu MI, Hutson JM: Cloacal and urogenital malformations in adriamycin-exposed rat fetuses. *BJU Int* 86:107–112 (2000).
- Liu MI, Hutson JM: Ontogeny of bladder agenesis in rats induced by adriamycin. *BJU Int* 87:556–561 (2001).
- Liu MI, Hutson JM, Zhou B: Critical timing of bladder embryogenesis in an adriamycin-exposed rat fetal model: a clue to the origin of the bladder. *J Pediatr Surg* 34:1647–1651 (1999).
- Logan CY, Nusse R: The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20:781–810 (2004).
- Lubinsky M: VATER and other associations: historical perspectives and modern interpretations. *Am J Med Genet Suppl* 2:9–16 (1986).
- Maeda Y, Dave V, Whitsett JA: Transcriptional control of lung morphogenesis. *Physiol Rev* 87:219–244 (2007).
- Mahlpuu M, Enerbäck S, Carlsson P: Haploinsufficiency of the forkhead gene *Foxf1*, a target for sonic hedgehog signaling, causes lung and foregut malformations. *Development* 128:2397–2406 (2001).
- Mallo M, Wellik DM, Deschamps J: *Hox* genes and regional patterning of the vertebrate body plan. *Dev Biol* 344:7–15 (2010).
- Masters JR: Epithelial-mesenchymal interaction during lung development: the effect of mesenchymal mass. *Dev Biol* 51:98–108 (1976).
- McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP: Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev* 12:1438–1452 (1998).
- Meller JL, Loeff DS, Reyes HM: A variant of the split notochord syndrome. *J Pediatr Surg* 24:733–735 (1989).
- Mendelsohn C, Lohnes D, Décimo D, Lufkin T, LeMeur M, et al: Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120:2749–2771 (1994).
- Merei J, Kotsios C, Hutson JM, Hasthorpe S: Histopathologic study of esophageal atresia and tracheoesophageal fistula in an animal model. *J Pediatr Surg* 32:12–14 (1997a).
- Merei J, Hasthorpe S, Farmer P, Hutson JM: Relationship between esophageal atresia with tracheoesophageal fistula and vertebral anomalies in mammalian embryos. *J Pediatr Surg* 33:58–63 (1998a).
- Merei JM: Embryogenesis of adriamycin-induced hindgut atresia in rats. *Pediatr Surg Int* 18:36–39 (2002).
- Merei JM: Single umbilical artery and the VATER-animal model. *J Pediatr Surg* 38:1756–1759 (2003).
- Merei JM: Notochord-gut failure of detachment and intestinal atresia. *Pediatr Surg Int* 20:439–443 (2004).
- Merei JM, Hutson JM: Embryogenesis of tracheoesophageal anomalies: a review. *Pediatr Surg Int* 18:319–326 (2002).
- Merei JM, Farmer P, Hasthorpe S, Qi BQ, Beasley SW, et al: Timing and embryology of esophageal atresia and tracheoesophageal fistula. *Anat Rec* 249:240–248 (1997b).
- Merei JM, Hasthorpe S, Farmer P, Hutson JM: Embryogenesis of tracheal atresia. *Anat Rec* 252:271–275 (1998b).
- Meriwether WD, Bachur NR: Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. *Cancer Res* 32:1137–1142 (1972).
- Miller MF, Cohen ED, Baggs JE, Lu MM, Hogenesch JB, Morrissey EE: Wnt ligands signal in a cooperative manner to promote foregut organogenesis. *Proc Natl Acad Sci USA* 109:15348–15353 (2012).
- Minoo P, Su G, Drum H, Bringas P, Kimura S: Defects in tracheoesophageal and lung morphogenesis in *Nkx2.1*($-/-$) mouse embryos. *Dev Biol* 209:60–71 (1999).
- Mollard R, Ghyselinck NB, Wendling O, Chambon P, Mark M: Stage-dependent responses of the developing lung to retinoic acid signaling. *Int J Dev Biol* 44:457–462 (2000).
- Momparler RL, Karon M, Siegel SE, Avila F: Effect of adriamycin on DNA, RNA, and protein synthesis in cell-free systems and intact cells. *Cancer Res* 36:2891–2895 (1976).
- Morrissey EE, Hogan BL: Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 18:8–23 (2010).
- Mortell A, O'Donnell AM, Giles S, Bannigan J, Puri P: Adriamycin induces notochord hypertrophy with conservation of sonic hedgehog expression in abnormal ectopic notochord in the adriamycin rat model. *J Pediatr Surg* 39:859–863 (2004a).
- Mortell A, O'Donnell AM, Giles S, Bannigan J, Puri P: Increased fibronectin expression in developing embryos is associated with abnormal notochord in the adriamycin rat model. *Pediatr Surg Int* 20:276–282 (2004b).
- Mortell A, Fourcade L, Solari V, Puri P: Bilateral megacreters in the Adriamycin rat model. *Pediatr Surg Int* 21:212–216 (2005).
- Motoyama J, Liu J, Mo R, Ding Q, Post M, Hui CC: Essential function of *Gli2* and *Gli3* in the formation of lung, trachea and oesophagus. *Nat Genet* 20:54–57 (1998).
- Murray CL, Reichert JA, Anderson J, Twigg LB: Multimodal cancer therapy for breast cancer in the first trimester of pregnancy. A case report. *JAMA* 252:2607–2608 (1984).
- Naiche LA, Papaioannou VE: Loss of *Tbx4* blocks hindlimb development and affects vascularization and fusion of the allantois. *Development* 130:2681–2693 (2003).
- Naito Y, Kimura T, Aramaki M, Izumi K, Okada Y, et al: Caudal regression and tracheoesophageal malformation induced by adriamycin: a novel chick model of VATER association. *Pediatr Res* 65:607–612 (2009).
- O'Bryan RM, Luce JK, Talley RW, Gottlieb JA, Baker LH, Bonadonna G: Phase II evaluation of adriamycin in human neoplasia. *Cancer* 32:1–8 (1973).
- O'Rahilly R, Müller F: Chevalier Jackson lecture. Respiratory and alimentary relations in staged human embryos. New embryological data and congenital anomalies. *Ann Otol Rhinol Laryngol* 93:421–429 (1984).
- Orford J, Manglick P, Cass DT, Tam PP: Mechanisms for the development of esophageal atresia. *J Pediatr Surg* 36:985–994 (2001).
- Orford JE, Cass DT: Dose response relationship between adriamycin and birth defects in a rat model of VATER association. *J Pediatr Surg* 34:392–398 (1999).
- Pedersen RN, Calzolari E, Husby S, Garne E; EUROCAT Working group: Oesophageal atresia: prevalence, prenatal diagnosis and associated anomalies in 23 European regions. *Arch Dis Child* 97:227–232 (2012).

- Peña A, Levitt MA: Anorectal anomalies, in Puri P, Höllwarth ME (eds): *Pediatric Surgery*, pp 291–312 (Springer, Heidelberg 2006).
- Pepicelli CV, Lewis PM, McMahon AP: Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr Biol* 8: 1083–1086 (1998).
- Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA: Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci USA* 99:10482–10487 (2002).
- Pitera JE, Smith VV, Thorogood P, Milla PJ: Co-ordinated expression of 3' *Hox* genes during murine embryonal gut development: an enteric Hox code. *Gastroenterology* 117:1339–1351 (1999).
- Pole RJ, Qi BQ, Beasley SW: Abnormalities of the tracheal cartilage in the rat fetus with tracheo-oesophageal fistula or tracheal agenesis. *Pediatr Surg Int* 17:25–28 (2001).
- Possoegael AK, Diez-Pardo JA, Morales C, Navarro C, Tovar JA: Embryology of esophageal atresia in the adriamycin rat model. *J Pediatr Surg* 33:606–612 (1998).
- Possoegael AK, Diez-Pardo JA, Morales C, Tovar JA: Notochord involvement in experimental esophageal atresia. *Pediatr Surg Int* 15:201–205 (1999).
- Qi B, Diez-Pardo JA, Navarro C, Tovar JA: Narrowing the embryologic window of the adriamycin-induced fetal rat model of esophageal atresia and tracheoesophageal fistula. *Pediatr Surg Int* 11:444–447 (1996).
- Qi B, Soto C, Diez-Pardo JA, Tovar JA: An experimental study on the pathogenesis of gastroesophageal reflux after repair of diaphragmatic hernia. *J Pediatr Surg* 32:1310–1313 (1997a).
- Qi BQ, Beasley SW: Preliminary evidence that cell death may contribute to separation of the trachea from the primitive foregut in the rat embryo. *J Pediatr Surg* 33:1660–1665 (1998).
- Qi BQ, Beasley SW: Pathohistological study of adriamycin-induced tracheal agenesis in the fetal rat. *Pediatr Surg Int* 15:17–20 (1999a).
- Qi BQ, Beasley SW: Communicating bronchopulmonary foregut malformations in the adriamycin-induced rat model of esophageal atresia. *Aust N Z J Surg* 69:56–59 (1999b).
- Qi BQ, Beasley SW: Relationship of the notochord to foregut development in the fetal rat model of esophageal atresia. *J Pediatr Surg* 34:1593–1598 (1999c).
- Qi BQ, Beasley SW: Stages of normal tracheo-bronchial development in rat embryos: resolution of a controversy. *Dev Growth Differ* 42:145–153 (2000).
- Qi BQ, Merei J, Farmer P, Hasthorpe S, Hutson JM, et al: Tracheomalacia with esophageal atresia and tracheoesophageal fistula in fetal rats. *J Pediatr Surg* 32:1575–1579 (1997b).
- Qi BQ, Merei J, Farmer P, Hasthorpe S, Myers NA, et al: The vagus and recurrent laryngeal nerves in the rodent experimental model of esophageal atresia. *J Pediatr Surg* 32:1580–1586 (1997c).
- Qi BQ, Merei J, Farmer P, Hasthorpe S, Myers NA, et al: Cardiovascular malformations in rat fetuses with oesophageal atresia and tracheo-oesophageal fistula induced by adriamycin. *Pediatr Surg Int* 12:556–564 (1997d).
- Qi BQ, Beasley SW, Williams AK: Evidence of a common pathogenesis for foregut duplications and esophageal atresia with tracheo-oesophageal fistula. *Anat Rec* 264:93–100 (2001).
- Qi BQ, Beasley SW, Frizelle FA: Evidence that the notochord may be pivotal in the development of sacral and anorectal malformations. *J Pediatr Surg* 38:1310–1316 (2003).
- Que J, Choi M, Ziel JW, Klingensmith J, Hogan BL: Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. *Differentiation* 74:422–437 (2006).
- Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, et al: Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 134:2521–2531 (2007).
- Rajagopal J, Carroll TJ, Guseh JS, Bores SA, Blank LJ, et al: Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development* 135:1625–1634 (2008).
- Rittler M, Paz JE, Castilla EE: VACTERL association, epidemiologic definition and delineation. *Am J Med Genet* 63:529–536 (1996).
- Rosenthal N, Brown S: The mouse ascending: perspectives for human-disease models. *Nat Cell Biol* 9:993–999 (2007).
- Sakiyama J, Yamagishi A, Kuroiwa A: *Tbx4-Fgf10* system controls lung bud formation during chicken embryonic development. *Development* 130:1225–1234 (2003).
- Sasaki T, Kusafuka T, Okada A: Analysis of the development of normal foregut and tracheoesophageal fistula in an adriamycin rat model using three-dimensional image reconstruction. *Surg Today* 31:133–139 (2001).
- Sato H, Murphy P, Giles S, Bannigan J, Takayasu H, Puri P: Visualizing expression patterns of *Shh* and *Foxf1* genes in the foregut and lung buds by optical projection tomography. *Pediatr Surg Int* 24:3–11 (2008).
- Shannon JM, Nielsen LD, Gebb SA, Randell SH: Mesenchyme specifies epithelial differentiation in reciprocal recombinants of embryonic lung and trachea. *Dev Dyn* 212:482–494 (1998).
- Sharpe J, Ahlgren U, Perry P, Hill B, Ross A, et al: Optical projection tomography as a tool for 3D microscopy and gene expression studies. *Science* 296:541–545 (2002).
- Shaw-Smith C: Oesophageal atresia, tracheo-oesophageal fistula, and the VACTERL association: review of genetics and epidemiology. *J Med Genet* 43:545–554 (2006).
- Shaw-Smith C: Genetic factors in esophageal atresia, tracheo-esophageal fistula and the VACTERL association: roles for *FOXF1* and the 16q24.1 FOX transcription factor gene cluster, and review of the literature. *Eur J Med Genet* 53:6–13 (2010).
- Shu W, Jiang YQ, Lu MM, Morrissey EE: Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129:4831–4842 (2002).
- Si-Tayeb K, Lemaigre FP, Duncan SA: Organogenesis and development of the liver. *Dev Cell* 18:175–189 (2010).
- Solomon BD: VACTERL/VATER association. *Orphanet J Rare Dis* 6:56 (2011).
- Spilde TL, Bhatia AM, Marosky JK, Hembree MJ, Kobayashi H, et al: Complete discontinuity of the distal fistula tract from the developing gut: direct histologic evidence for the mechanism of tracheoesophageal fistula formation. *Anat Rec* 267:220–224 (2002a).
- Spilde TL, Bhatia AM, Miller KA, Ostlie DJ, Chaignaud BE, et al: Thyroid transcription factor-1 expression in the human neonatal tracheoesophageal fistula. *J Pediatr Surg* 37: 1065–1067 (2002b).
- Spilde TL, Bhatia A, Ostlie D, Marosky J, Holcomb G 3rd, et al: A role for sonic hedgehog signaling in the pathogenesis of human tracheoesophageal fistula. *J Pediatr Surg* 38: 465–468 (2003a).
- Spilde TL, Bhatia AM, Marosky JK, Preuett B, Kobayashi H, et al: Fibroblast growth factor signaling in the developing tracheoesophageal fistula. *J Pediatr Surg* 38:474–477 (2003b).
- Spilde TL, Bhatia AM, Mehta S, Ostlie DJ, Hembree MJ, et al: Defective sonic hedgehog signaling in esophageal atresia with tracheoesophageal fistula. *Surgery* 134:345–350 (2003c).
- Spilde TL, Bhatia AM, Mehta SS, Hembree MJ, Preuett BL, et al: Aberrant fibroblast growth factor receptor 2 signalling in esophageal atresia with tracheoesophageal fistula. *J Pediatr Surg* 39:537–539 (2004).
- Spitz L: Oesophageal atresia. *Orphanet J Rare Dis* 2:24 (2007).
- Spitz L, Kiely EM, Morecroft JA, Drake DP: Oesophageal atresia: at-risk groups for the 1990s. *J Pediatr Surg* 29:723–725 (1994).
- Stankiewicz P, Sen P, Bhatt SS, Storer M, Xia Z, et al: Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of *FOXF1* cause alveolar capillary dysplasia and other malformations. *Am J Hum Genet* 84:780–791 (2009).
- Straaten HW, Hekking JW, Wiertz-Hoessels EJ, Thors F, Drukker J: Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. *Anat Embryol* 177:317–324 (1988).
- Summerhust K, Stark M, Sharpe J, Davidson D, Murphy P: 3D representation of Wnt and frizzled gene expression patterns in the mouse embryo at embryonic day 11.5 (Ts19). *Gene Expr Patterns* 8:331–348 (2008).

- Sutliff KS, Hutchins GM: Septation of the respiratory and digestive tracts in human embryos: crucial role of the tracheoesophageal sulcus. *Anat Rec* 238:237–247 (1994).
- Szumaska D, Pieles G, Essalmani R, Bilski M, Mesnard D, et al: VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase *Pcsk5*. *Genes Dev* 22:1465–1477 (2008).
- Tam PP, Kanai-Azuma M, Kanai Y: Early endoderm development in vertebrates: lineage differentiation and morphogenetic function. *Curr Opin Genet Dev* 13:393–400 (2003).
- Tan C, Etcubanas E, Wollner N, Rosen G, Gylladoga A, et al: Adriamycin—an antitumor antibiotic in the treatment of neoplastic diseases. *Cancer* 32:9–17 (1973).
- Temelcos C, Hutson JM: Ontogeny of the VATER kidney in a rat model. *Anat Rec A Discov Mol Cell Evol Biol* 278:520–527 (2004).
- Tepass U: The apical polarity protein network in *Drosophila* epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. *Annu Rev Cell Dev Biol* 28:655–685 (2012).
- Tepass U, Truong K, Godt D, Ikura M, Peifer M: Cadherins in embryonic and neural morphogenesis. *Nat Rev Mol Cell Biol* 1:91–100 (2000).
- Thesleff I, Vaahtokari A, Partanen AM: Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int J Dev Biol* 39:35–50 (1995).
- Thompson DJ, Molello JA, Strebing RJ, Dyke IL: Teratogenicity of adriamycin and daunomycin in the rat and rabbit. *Teratology* 17:151–157 (1978).
- Tomaselli V, Volpi ML, Dell’Agnola CA, Bini M, Rossi A, Indriolo A: Long-term evaluation of esophageal function in patients treated at birth for esophageal atresia. *Pediatr Surg Int* 19:40–43 (2003).
- Tugay M, Filiz S, Dalçik H, Güvenç BH, Dalçik C, et al: Expression of cell adhesion molecules in the adriamycin-induced esophageal atresia rat model. *Cell Biol Int* 27:929–933 (2003a).
- Tugay M, Yildiz F, Utkan T, Sarioglu Y, Gacar N: Gastric smooth muscle contractility changes in the esophageal atresia rat model: an in vitro study. *J Pediatr Surg* 38:1366–1370 (2003b).
- Ukita K, Hirahara S, Oshima N, Imuta Y, Yoshimoto A, et al: Wnt signaling maintains the notochord fate for progenitor cells and supports the posterior extension of the notochord. *Mech Dev* 126:791–803 (2009).
- van Heurn LW, Cheng W, de Vries B, Saing H, Jansen NJ, et al: Anomalies associated with oesophageal atresia in Asians and Europeans. *Pediatr Surg Int* 18:241–243 (2002).
- Warburton D, Bellusci S: The molecular genetics of lung morphogenesis and injury repair. *Paediatr Respir Rev* 5 Suppl A:S283–S287 (2004).
- Weaver M, Dunn NR, Hogan BL: Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* 127:2695–2704 (2000).
- Weaver M, Batts L, Hogan BL: Tissue interactions pattern the mesenchyme of the embryonic mouse lung. *Dev Biol* 258:169–184 (2003).
- Wells JM, Melton DA: Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 15:393–410 (1999).
- Williams AK, Qi BQ, Beasley SW: Temporospatial aberrations of apoptosis in the rat embryo developing esophageal atresia. *J Pediatr Surg* 35:1617–1620 (2000).
- Williams AK, Qi BQ, Beasley SW: Demonstration of abnormal notochord development by three-dimensional reconstructive imaging in the rat model of esophageal atresia. *Pediatr Surg Int* 17:21–24 (2001).
- Williams AK, Quan QB, Beasley SW: Three-dimensional imaging clarifies the process of tracheoesophageal separation in the rat. *J Pediatr Surg* 38:173–177 (2003).
- Williamson KA, Hever AM, Rainger J, Rogers RC, Magee A, et al: Mutations in *SOX2* cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum Mol Genet* 15:1413–1422 (2006).
- Wilson JG, Roth CB, Warkany J: An analysis of the syndrome of malformations induced by maternal vitamin a deficiency. Effects of restoration of vitamin a at various times during gestation. *Am J Anat* 92:189–217 (1953).
- Xia H, Migliazza L, Montedonico S, Rodriguez JI, Diez-Pardo JA, Tovar JA: Skeletal malformations associated with esophageal atresia: clinical and experimental studies. *J Pediatr Surg* 34:1385–1392 (1999).
- Xiaomei L, Aras-Lopez R, Martinez L, Tovar JA: Lung hypoplasia in rats with esophageal atresia and tracheo-esophageal fistula. *Pediatr Res* 71:235–240 (2012).
- Yang SZ, Kohno N, Kondo K, Yokoyama A, Hamada H, et al: Adriamycin activates E-cadherin-mediated cell-cell adhesion in human breast cancer cells. *Int J Oncol* 15:1109–1115 (1999).
- Yoo HJ, Kim WS, Cheon JE, Yoo SY, Park KW, et al: Congenital esophageal stenosis associated with esophageal atresia/tracheoesophageal fistula: clinical and radiologic features. *Pediatr Radiol* 40:1353–1359 (2010).
- Young RC, Ozols RF, Myers CE: The anthracycline antineoplastic drugs. *N Engl J Med* 305:139–153 (1981).
- Zaw-Tun HA: The tracheo-esophageal septum – fact or fantasy? Origin and development of the respiratory primordium and esophagus. *Acta Anat* 114:1–21 (1982).
- Zeng X, Goetz JA, Suber LM, Scott WJ Jr, Schreiner CM, Robbins DJ: A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* 411:716–720 (2001).
- Zhou B, Hutson JM, Farmer PJ, Hasthorpe S, Myers NA, Liu M: Apoptosis in tracheoesophageal embryogenesis in rat embryos with or without adriamycin treatment. *J Pediatr Surg* 34:872–875 (1999).