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Review

Embryonic transplantation experiments: Past, present, and future

Grace E. Solini, Chen Dong and Margaret Saha*

Biology Department, The College of William and Mary, Integrated Science Center, 540 Landrum Dr., Williamsburg, VA 23185, USA.

ABSTRACT

The process of taking a piece of tissue and transplanting it into a novel location has been of paramount importance for life sciences. The technique of transplantation has served an important role in providing a basic understanding of all facets of biology ranging from cancer and evolutionary biology to developmental biology. First employed by early embryologists, transplantation has played a particularly critical role in elucidating virtually every aspect of embryonic development including cell specification, commitment, cell fate determination, embryonic induction, and plasticity. This review will detail the essential role cell transplantation experiments have played in uncovering fundamental developmental and cell biological processes as well as their valuable contribution to contemporary developmental biology. Finally, it will suggest fruitful directions that this technique, in conjunction with current molecular and sequencing technologies, could play in future work.

KEYWORDS: transplantation, transplant, graft, embryo, plasticity

INTRODUCTION

The technique of embryonic tissue transplantation has had a long and productive history. The widespread use of embryonic tissue transplantation dates back to the nineteenth century when a new generation of embryologists attempted to move beyond anatomical observations and make embryology an experimental rather than a descriptive science. Practitioners of what was termed Entwicklungsmechanik attempted to uncover the mechanisms governing development by physically manipulating the embryo and assaying downstream effects on development [1]. A major tool in their arsenal was the technique of tissue transplantation in which investigators transferred a section of embryonic tissue from a donor embryo onto a new host embryo or back onto the donor embryo itself. The transfer could involve placing the tissue into the same (iso- or homotopic) or different (heterotopic) location, and/or positioning the tissue into an embryo of the same (iso- or homochronic) or different (heterochronic) age. Transplants could also be placed in the same orientation or rotated, and even be placed into the same or different species, with each of these varied manipulations addressing different questions. Using tissue transplantation approaches, these early experimental embryologists not only made considerable strides in elucidating causal relationships in development, but their work also led to the important conceptual advances and the articulation of key embryological concepts such as determination, competence and induction [1]. However, despite the importance of this early work, progress was limited without availability of techniques for decisive host and donor marking and in the absence of the approaches made possible by the knowledge of modern molecular genetics and cell biology. With the advent of tissue-specific genetic markers and the ability to detect and manipulate gene expression, a new wave of findings has emerged from

*Corresponding author: msaaha@wm.edu

transplantation experiments specifically related to determination and competence, inducing capacity, fate-mapping, plasticity, and the function of key embryonic genes. This article will review some of the key discoveries and insights provided by the broad range of transplantation experiments performed during early embryonic development and will conclude with possible avenues for future research.

Determination and competence of embryonic tissues

Adoption of a particular cell fate is a multistep process during which cells first become specified, which means they will adopt a cell identity that is in accord with a fate map when developing in isolation in a neutral environment, but are still competent to respond to signals and change their fate if moved to a different region of the embryo. The specified cells then become determined, maintaining their fated identity even when placed in a different embryonic environment [2]. While tissue explants have provided information on the state of specification, transplantation has served as an essential technique to study the state of determination of tissues during various stages of development. Determination is inextricably associated with the competence of a tissue, that is, the ability of a tissue to respond to a specific set of inductive signals. If a tissue is not able to respond to inductive signals from the environment (the competence of the tissue), the tissue will retain its state of determination at the time when it was removed from the host. Manipulating the location where the transplanted tissues are placed reveals the spatial distribution of states of commitment, while altering the time when transplants are made can reveal the temporal aspects of determination and competence of various tissues within an embryo.

One of the most widely studied and classic tissues for transplantation experiments is the neuroectoderm, beginning with the iconic studies of Spemann and his group showing that presumptive neural ectoderm from an early gastrula amphibian embryo will assume the fate of its host environment while presumptive neural tissue from a late gastrula will retain its neural fate [3]. These experiments have been repeated using molecular markers in a number of different species, and through a series of heterochronic transplants, researchers were able to conclude that the competence for ectodermal tissue to adopt a neural fate steadily declines as donor embryos age and by neural plate stages the neuroectoderm is largely determined to adopt a neural fate [4-8], indicating a gradual commitment of dorsal ectodermal cells for neural differentiation [9]. Neural crest tissue, on the contrary, is competent to respond to signals in its microenvironment until much later [10, 11]. More about the neuroectoderm will be discussed below in the induction and plasticity sections.

Transplantation experiments also addressed the state of determination in a wide array of other tissues including retina, and non-neural tissues such as notochord, prethalamus, hematopoietic system, anterior endoderm and the primary mouth [12-17]. Transplantation was also employed to address determination at very early stages of development. In zebrafish, the presumptive enveloping layer (EVL) cells are committed to an EVL exclusive fate by the late blastula stage [18]. As evidence for EVL commitment, when the EVL cells are transplanted heterotopically and heterochronically, they follow unconventional migration paths as a way of compensating to end up in the correct location [18]. Similar studies in *Xenopus laevis* showed that vegetal pole cells become committed to endoderm by the early gastrula stage [19, 20]. The animal pole cells, on the other hand, pass from pluripotency to a labile state of commitment to ectoderm during the blastula stage [21]. They become responsive to mesodermal induction at stage 6.5 and lose responsiveness around stage 10.5 [22]. However, transplantation of dorsal blastomere cell at 16- or 32-cell stage to a more ventral location either resulted in the formation of a secondary axis, or the repositioning of donor cell progenies to a more animal dorsal location [23, 24], indicating the ability to form future head organizer is established in dorsal equatorial blastomeres at stage 5 or 6 [25, 26]. There is some evidence suggesting the establishment of such ability at as early as 8-cell stage [27].

Tissue transplantation has been central for identifying the spatial and temporal aspects of when and which embryonic tissues become determined to adopt a placodal or sub-placodal fate, specifically the competence of placodal regions. Placodes are discrete regions of thickened ectoderm from which many cranial sense organs and ganglion including lens, ear, and nose arise [28, 29]. The formation of the eye lens

has served as a classic model for examining the competence of a tissue. Spemann first reported that most ectoderm during gastrulation possesses lens-forming competence, which was revealed after ectoderm from different parts of the embryo was transplanted to the presumptive eye region and formed lens tissue [30]. The result was corroborated by Henry and Grainger in a more extensive and stage-controlled study [31], when they transplanted various ectodermal tissues either into the lens-forming region of open neural plate stage host embryos or over the newly formed optic vesicle of somewhat later neurula stage embryos. Using unambiguous molecular markers, they showed that most non-neural ectodermal tissues have some lens-forming potential during early gastrula stages, but that this potential becomes restricted to the presumptive lens-forming region and closely adjacent regions throughout neurula stages [31]. After transplanting animal cap ectoderm to the presumptive lens area, they also discovered that this early gastrula ectoderm has minimal lens-forming competence but possesses considerable neural competence [32]. However, as ectoderm is taken from embryos of increasing age, neural competence is lost and competence to form a lens is acquired [32]. A later ectoderm-to-lens transplantation experiment coupled with *in situ* hybridization of marker genes demonstrated the linkage between the expression of two genes, *Otx-2* and *Pax-6*, and the competence of presumptive lens tissues to respond to lens-inducing signals [33]. Integrating transplantation and molecular techniques, another study on zebrafish that transplanted cells with activated Hedgehog signaling cascade to blastula stage hosts found that overexpression of the Hedgehog signaling pathway blocks lens formation and induces ectopic pituitary gene expression in a non-autonomous fashion [34].

In addition to the lens, transplantation experiments have also addressed determination and competence in other placode-derived structures. By heterotopically and heterochronically grafting ectoderm to the presumptive olfactory placodal region in a quail-chick chimera system, followed by assaying the expression of olfactory placodal marker genes, Bhattacharyya and Bronner-Fraser showed that competence to form olfactory placode resides within head ectoderm up until HH 9-10, but is largely absent from trunk ectoderm; olfactory fate specification and commitment are complete just before acquiring overt placodal morphology and prior to differentiation into olfactory epithelium at HH14 [35]. Experiments in amphibians showed similar results [29]. Studies on competence and specification of the ophthalmic trigeminal (opV) placode found that the whole head ectoderm rostral to the first somite is competent to form opV placode when grafted to the opV placode region at 3-somite stage, though competence is rapidly lost thereafter in otic-level ectoderm and determination is complete by the 8-somite stage [28]. More focused research on specification and determination of neuronal phenotypes within the presumptive placodal ectoderm also employed transplantation techniques [36]. The results suggested that at E1.5 the tissues are far from determined; non-neurogenic ectoderm from the trunk was competent to differentiate into nodose-type neurons when transplanted heterotopically to the nodose region of the placode [36]. Taken together, these experiments demonstrated the integral role transplantation plays in understanding the temporal and spatial boundaries of competence and the state of determination of various tissues within embryos.

Inductive ability of embryonic tissue

While the competence of embryonic tissue is extremely important, the inducing signal itself plays an equally critical role in cell fate commitment; therefore the inductive ability of embryonic tissue has also received enormous attention in developmental biology. Transplantation has served as an effective technique to address the problem. By grafting donor tissues at various time points and into various locations near the host tissue that is known to be competent, the host response could reveal the potency as well as the spatial and temporal limit of the inducing signal.

Perhaps the most famous transplantation experiment in the history of developmental biology is the organizer experiment [37]. Following the transplantation of the dorsal lip region (organizer) at early gastrula stage to the ventral region of another amphibian embryo, the donor organizer induces the formation of a secondary embryo [37]. This was the first time that a specific embryonic tissue was shown to possess the ability to induce neural fate and organize a new, duplicated axis on

the ventral side of the embryo. A similar experiment was performed on avian embryos by Waddington several years later, revealing that the anterior region of the primitive streak, known as the Hensen's node, has the same neural inducing ability as the amphibian organizer [38]. The shield region in fish was discovered to be analogous to the organizer [39]. While the mouse node was long thought to possess the organizer ability, it wasn't experimentally confirmed until 1994 [40]. In amphibians, the organizer itself is shown to be induced by the Nieuwkoop center at the dorsal vegetal pole, as transplanted dorsal vegetal blastomeres are capable of axis induction [41]. This ability is likely the result of dorsal-inducing material in the cytoplasm near the vegetal cortex [42-44]. Organizer tissue largely gives rise to mes-endodermal tissues while the resulting neuroectoderm is mainly of host origin [37, 40], though in zebrafish it was reported that the organizer graft contributes to the ectopic neuraxis [39]. The inductive ability of the organizer is largely dependent on age, as the frequency of neural induction declines with advancing donor age [5].

The age of the organizer is an important determinant of the type of neural tissue induced. In chick embryos, young nodes (HH stages 2-4) are capable of inducing both anterior and posterior neural structures, while older nodes (HH stages 5-6) tend to have less overall inducing ability and are able to give rise to posterior nervous system [8]. This is consistent with the Nieuwkoop activation-transformation hypothesis, where a wave of activation initially induces the presumptive neuroectoderm to an anterior neural fate, and a transforming factor then posteriorizes the already neuralized tissue [45]. Surprisingly, one study found that the chordoneural hinge and the tip of the tail retain Spemann's tail organizer activity even during tadpole stages of development [46]. Molecular analysis revealed that the "inducing capacity" of the organizer was actually attributable to the inhibition of the bone morphogenetic protein (BMP) pathway by BMP-inhibiting factors within the organizer such as chordin, noggin, and follistatin [47-51]. The dependency of organizer's dorsalizing ability on these factors was demonstrated using transplantation combined with molecular techniques. When chordin expression in donor embryo is knocked down, the organizer failed to induce neural tissue in host embryo [52]. The importance of chordin was further demonstrated when embryos transplanted with Spemann organizer tissue in the ventral side produced a second gradient of Chordin [53]. However, while they are required for neural induction by the organizer, they do need to work synergistically with other pathways to induce neural fate in competent ectoderm. In quail-chick chimeras, it was shown that chordin is sufficient to initiate primitive streak, but not neural tissue in competent epiblast [54]. Another important contributor to neural induction is the fibroblast growth factor (FGF) signaling pathway, since a defective FGF pathway was shown to severely interfere with induction [55].

The various transplantation experiments led to widespread acceptance of the ability of organizers to induce neural tissue. However, other tissues have been proposed to possess the ability of neural induction, though these views are more controversial. One such idea is the homeogenetic induction of neural tissue, which refers to the induction of neural tissue by existing neuroectoderm. This phenomena was first reported by Waddington in avians, when a neural plate graft was able to induce host neural plate formation [56]. This ability was further demonstrated in *Xenopus* when the presumptive neural plate of late gastrulae induced further neural structures in competent early gastrula ectoderm [32, 57]. Besides inducing the general neuroectoderm, homeogenetic induction has also been shown to contribute to the regionalization of the embryonic nervous system. Transplanted neural plate tissue was able to induce hatching gland and cement gland formation close to host neuroectoderm [58]. When presumptive chick neural tube tissue is transplanted adjacent to presumptive epidermis, neural crest cells can arise from both tissues [59] and when the prethalamic precursors were transplanted to areas of the future forebrain, they induced ectopic prethalamic marker gene expression at the surrounding host forebrain [17]. Interestingly, one study found that organizer transplants are able to induce both neural tissue and its surrounding epidermis, which is marked by DLX5 expression, and the inductive effect is transferred *via* the neural plate to the periphery [60]. This supported the view that there

are planar signals between dorsal neuroectoderm and ventral epidermis.

In addition to organizer and homeogenetic induction, the endoderm was also proposed to have neural inductive ability. Waddington observed that rotating the endoderm typically impeded the lengthening of the primitive streak, or triggered the development of a new primitive streak in chicks [6, 61]. However, it wasn't completely clear if this was caused by the neural inductive ability of the endoderm or simply the ability for endoderm to induce cell movement that leads to the development of the primitive streak [6]. Both camps have found evidence that support their own argument. After rotation of the hypoblast by 90° in chicks at stage XIII, the direction of the primitive streak was according to the orientation of the hypoblast, whereas at HH stage 3, it gradually shifted towards that of the epiblast, thus identifying a window of hypoblast inductive ability [62]. Another endodermal tissue, the prechordal plate, also showed anterior neural inductive ability [63]. It was even discovered that there is a correlation between the degree to which transplanted organizer cells contributed to the host endoderm and the frequency of neural induction [5]. However, there is also a body of evidence arguing against endoderm as a neural inducer. In contrast to the results of Waddington and Azar and Eyal-Giladi, other investigators did not observe that the rotated hypoblast of the chicken embryo gives rise to an ectopic axis in the epiblast. Rather, they concluded that the embryonic axis simply develops according to the basic polarity of the epiblast layer [64]. Such finding was reinforced by Foley, Skromne and Stern [65] who argued that hypoblast isn't a head organizer because it neither induced neural tissue from naive epiblast nor changed the regional identity of neural tissue, and its real role is directing cell movements in the adjacent epiblast, echoing Waddington's initial hypothesis [38, 61]. It is worth noting that in one study, hypoblast from rabbit but not chick embryo is able to induce neural tissue [66]. Additional studies will elucidate the precise role endoderm plays in neural induction.

Despite most research on the inductive ability of embryonic tissues focusing on neural induction, the induction of other tissues and structures during embryonic development has also been extensively studied. The induction of lens, neural crest, notochord, placode, floor plate, primary mouth, Rathke's pouch, and even the three germ layers have all been addressed using transplantation techniques [13, 21, 31, 67-75]. Combined with other techniques, transplantation has significantly increased our understanding of the inductive ability of embryonic tissues during early development.

Embryonic plasticity

An essential aspect of embryonic development is plasticity, which refers to the ability to respond to continual perturbations in order to adjust to changing - sometimes adverse - conditions [76]. While several different forms of perturbation exist, including physical, chemical, genetic, and extreme temperature, transplantation has become one of the most common ways to understand embryonic plasticity following physical perturbation, as it allows the study of plasticity on both the tissue level and the individual cellular level. Plasticity is quite obviously related to the state of determination given that determined tissues will fail to show regulation. However the focus of these studies is on the temporal and spatial aspects of the plasticity itself and the mechanisms governing the ability of the tissue or cells to easily alter their fate in response to environmental conditions. With the burgeoning field of regenerative medicine, there has been heightened interest in plasticity.

Though most types of embryonic tissue possess some degree of plasticity, transplantation studies have been primarily focused on the plasticity of anterior-posterior (AP) neural axis as well as that of neuronal innervation. Earlier embryological studies on AP neural axis plasticity examined the plasticity of the entire neuroectoderm. The main technique employed was the 180 degrees rotation transplant of the neuroectoderm along the AP axis of the embryo. By assaying the recovery of the transplant embryo, as well as the regional identity of transplant tissue at a later stage, the degree of AP neural plasticity can be revealed. If the anterior-posterior identity of cells in the transplant tissue follows the AP pattern of the host, it suggests that the AP characteristics in the donor neuroectoderm was not yet determined; on the other hand, if cells in the rotated tissue adopt anterior-posterior identity according to their original AP orientation,

it is likely that the donor embryo has lost its AP neural axis plasticity. This period during which the neuroectoderm is competent to respond to signals from host tissue and adopt new AP regional identity is referred to as the window of plasticity. When Spemann performed AP neural axis rotation for the first time, he found that neural plate stage embryos have lost their AP neural plasticity [77, 78]. However, this result should be treated with caution because the inductive ability of mesoderm wasn't fully understood at the time, and Spemann rotated the neuroectoderm along with the underlying mesoderm.

When scientists started to rotate only the dorsal ectoderm, two opposing results initially emerged. Some found that at neural plate stage, rotation of the neuroectoderm caused the neural plate to develop in a completely reverse orientation, and therefore drew the conclusion that AP axis is already established and plasticity is lost at that stage [79]. Others also rotated the neuroectoderm, but saw complete or mostly complete recovery of the transplant embryo and argued that AP neural axis plasticity is still present at neural plate stage [80, 81]. It turns out that such differences were due to the different sizes of the rotated transplanted tissue. When the size of the rotated graft increases, the level of AP neural axis dysregulation worsens [82]. When the size of the rotated graft was taken into consideration, it was discovered that the patterning of the AP axis is relatively fixed by the neural plate stage [82]. The transplantation of *Xenopus* prospective spinal neuroectoderm tissue to presumptive eye and prosencephalic regions at neural plate stage found a mixture of anterior and posterior features at the transplant regions [83], while the transplantation of anterior neural plate to more posterior positions showed full caudalization at stage 11/12 and no caudalization at stage 16 [7], thus corroborating this conclusion. Additional rotation transplant of the organizer and neuroectoderm done at earlier stages revealed the presence of AP neural axis plasticity at early gastrula stage [84]. In conjunction with other experiments, a window of AP plasticity was identified between early to mid-gastrula and neural plate stage [85, 86]. But since these transplantations were done prior to the age of unambiguous lineage tracers and genetic markers, a study on the AP neural axis plasticity of the entire neuroectoderm using transplantation and molecular techniques may provide us with much more insight into this topic.

The availability of specific, highly localized regional markers allowed investigators to focus on much smaller regions of neuroectodermal axis. Thanks to the discrete expression patterns of many Hox genes within individual rhombomeres, thus making them perfect candidates of regional marker genes, many transplantation experiments have been performed to investigate the AP neural axis plasticity of the hindbrain rhombomeric region [74]. A specific rhombomere is transplanted to an ectopic location along the AP neural axis, and the ability of the donor tissue to activate Hox gene expression unique to the new location and turn off expression of marker gene specific to the donor site serves as an indicator of the level of AP plasticity. The results of these studies have revealed a complex amalgam of plasticity and autonomy, depending on both donor and host location, size of the graft, as well as stage [74, 87]. When transplants are performed at the anterior region of the embryonic neuroectoderm, rhombomeres generally don't exhibit much plasticity [74]. Numerous studies have shown that when grafted rostral to the otic vesicle, ectopic rhombomeres will express the Hox gene according to its original location as early as HH st. 9 [88-92]. On the other hand, when rhombomeric transplants were grafted caudal to the otic vesicle, they showed considerable level of plasticity [74]. Rostral to caudal rhombomere grafts were able to modify their Hox gene expression pattern in accordance to their new location [91, 93, 94]. This discrepancy could be explained by the finding that rhombomere inductive signal is distributed in a decreasing caudal-rostral gradient [94], as well as the discovery of a posteriorizing signal [95]. The idea that cells in different rhombomeres are intrinsically variable and respond to environmental signals differently may also help with the explanation [96].

The plasticity along the AP neural axis is hardly the only type of embryonic plasticity being studied using transplantation. Due to its significant implications in regenerative medicine, the plasticity of neuronal innervation has also received much scientific interest. While this topic has been extensively researched since the early twentieth century [97, 98], here we choose to review those that were done following the availability of molecular techniques.

When the neural tube of an early tailbud stage *Xenopus* embryo was rotated 180 degrees, observation of the axonal trajectories of KA neurons revealed that while a few neurons reoriented to follow the AP axis of the host, most neurons within the transplant followed the original orientation of the graft, indicating that the plasticity of axonal trajectories has been lost at this point in development [99]. The neurons in otic placode showed a higher degree of innervative plasticity. When the presumptive ear region was transplanted posteriorly to replace a somite at mid-tailbud stage, the transplanted ears could develop relatively normally with complete efferent and afferent innervations to and from the spinal cord [100]. In fact, while there is some plasticity inherent to motor innervation, otic placode was proposed to be the only tissue that can be innervated by all motor neurons [101]. Additional transplantation experiments revealed that the window of motor neuron innervative plasticity was determined to be between stage 14 and 15 [102], while the plasticity of rostral-caudal positional identity of motor neurons persists until neural tube closure [103, 104]. The motor neuron identity was also shown to be determined before axogenesis [105]. It was further demonstrated that the motor neuron identity and migratory path is sensitive to positionally restricted signals from the paraxial mesoderm and rhombomeres, respectively [103, 106]. The plasticity of sensory neuron innervation is also well studied. It has been shown in frogs that when eye primordia is grafted along the body axis to create ectopic eyes during tailbud stage, it is able to undergo sensory neuron innervation and confer vision to the host [107]. Further research showed that serotonergic stimulation can significantly enhance the innervation efficiency of sensory neurons [108]. This corroborates the idea that the initial guidance for afferent innervation of sensory neurons is based upon neurotransmitter signaling; these connections are then subsequently fine-tuned through an activity-based mechanism [101]. The plasticity of neuronal innervation has also been shown to depend on short-range cues, as in the case of posterior cement gland signaling the innervation of the mandibular trigeminal nerve when rotated or transplanted ectopically [109].

Fate mapping of early embryonic tissue

Tissue transplantation has also served as an invaluable tool for studying the movements and fates of tissues throughout development. This form of fate mapping often involves isotopic and homochronic transplantation of tissue from donor to host, and it is advantageous because donor cells and their derivatives can be easily distinguished from host cells, facilitating an accurate mapping of transplanted tissue fate. Among the most well studied tissue systems in developmental biology is the embryonic neuroectoderm. Transplantation studies have been utilized to elucidate both general and detailed information regarding the developmental fate of various neural structures. The neural crest, for instance, has been a well-studied structure in various organisms and in a variety of detail. Rosenquist utilized transplantation methods in labeled chick embryos to confirm the origin of neural crest cells as the neural/non-neural border in the anterior epiblast [110]. Later work in *Xenopus* embryos has mapped neural crest cells in more detail, using interspecies transplants of *Xenopus borealis* donor cells within host *Xenopus laevis* embryos to study different segments of the neural crest and their derivatives. The initial findings of this research revealed that the mandibular crest migrates along two pathways, the first contributing to the lower jaw, quadrate, and ethmoid-trabecular cartilages, and the second to the trigeminal ganglia, the cornea, connective tissues, and the mesenchymal and choroid layers of the eye. The branchial crest consists of two segments contributing to the cartilages of the gills, and the hyoid crest contributes to the formation of ceratohyal cartilages and the muscles connected to the ceratohyal cartilage [111]. These discoveries allowed researchers to conclude that even in early developmental stages the neuroectoderm is highly organized, as each separate segment of the neural crest is spatially organized and contributes to structures of similar function. Similar transplants of presumptive neural crest cells as well as mesoderm transplants performed orthotopically in mice has indicated that the branchial arch and other craniofacial structures such as the facial, cervical, peri-ocular, and peri-otic mesenchyme derive from the cranial paraxial mesoderm as well the neural crest cells. In more dorsal craniofacial regions, this was observed as a co-distribution of mesoderm and neural crest cells, while more ventral regions such as the branchial arches exhibited a segregation of the two populations [112]. In addition to those of the neural crest, the origins and

derivations of the neural plate and ridge have also been extensively studied using transplant fate mapping techniques. In one study, a comprehensive map of the location and dimensions of the prospective forebrain, hindbrain, midbrain, and spinal cord in the chick neural plate was determined by superimposing overlapping fate maps from transplants of different regions spanning the neural plate [113]. Early studies by Eagleson and Harris utilized DiI-labeled transplants of the neural plate and neural ridge to map the presumptive brain regions of *Xenopus laevis*. Though most brain regions were expected to derive from the neural plate, the ventral forebrain, the dorsal brain stem, the anterior pituitary, and the telencephalon were found to arise only from neural ridge transplants [114].

Other transplantation studies on the anterior pituitary and telencephalon have mapped the origin and derivatives of these structures in greater detail. The ventral neural ridge, for instance, was elucidated as the origin of the anterior pituitary through isotopic-labeled donor transplants in *Xenopus laevis* [115]. Similar transplants of the neural ridge were done using quail-chick chimera transplants, revealing that the posterior pituitary originates in a region of the neural folds separate from the anterior pituitary and that these two regions are separated by the presumptive hypothalamus [116, 117]. Quail-chick chimera transplants were also utilized to perform transplants of the telencephalon to locate the telencephalic subpallium as the origin of inhibitory neurons invading the pallium, finding that 90% of GABAergic neurons in the pallium originate from the striatal and palliopetal regions of the telencephalic subpallium. Grafts of the anterior entopeduncular area within the subpallium also revealed tangential migration of oligodendraglial cells to the pallium in addition to the inhibitory neurons migrating from the striatum and pallidum [118]. Detailed mapping of these structures has facilitated a greater understanding of the origins and pathways of significant neural structures during development.

Within the research dedicated to mapping the fate of the neuroectoderm there has been a significant focus on the rhombencephalon, or the hindbrain. Divided into six rhombomeres (r1-r6) and five pseudorhombomeres ("r7"-"r11"), the rhombencephalon has received a lot of attention with regard to discerning the degree of organization as well as exclusivity of the different segments. Homochronic transplants from quail to chick embryos of the six rhombomeres have revealed that the cerebellum arises from the first two rhombomeres (r1-r2), the caudal pontine reticular nucleus of the reticular formation originates from the third and fourth rhombomeres (r3-r4), the cochlear nuclei derive from regions in r3 and r5-r7, the trigeminal column is formed from rhombomeres r2-r6 and pseudorhombomeres r7 and r8, the raphe region has origins in r3 as well as in r6-r7, and motor nuclei are found within all six of the rhombomeres [119]. These conclusions were modified slightly by a similar study on the pseudorhombomeres r7-r11. After isotopic quail-chick chimera grafts were utilized to study the fates of the r7-r11 regions, the prospective hindbrain region was extended from r1-r6 to include the five pseudorhombomeres, with the new prospective hindbrain now mapping the entire r1-r11 region. The region corresponding to the cochlear nuclei was extended to include r8 instead of the previously accepted boundary at r6/r7. The choiroidal roof and rhombic lip, in addition to being present in rhombomeres r1-r6, was also found in the r7-r11 region, extending the presence of these structures to a general property of both rhombomeres and pseudorhombomeres [120]. In a more detailed study, the relationship between rhombomeres and vestibular neuron populations was determined by transplants of rhombomeres r3, r4, and r5 from quail to chick embryos and tracing of axonal projections through biotinylated dextran amine (BDA) labelling of neurons. Tracing of the vestibulospinal groups revealed that each group is localized to a single rhombomere, each with a unique set of axon projection phenotypes. The lateral vestibulospinal tract is localized in r4, though there is moderate spillover into r5, the contralateral vestibulospinal tract originates in r5, and the ipsilateral vestibulospinal tract is localized in r6. Of the vestibular-ocular groups, two groups were found to span across two rhombomeres, with the contralateral rostral group spanning r1-r2 and ipsilateral rostral spanning r2-r3; the ipsilateral caudal localized a single rhombomere and was found within r5. The contralateral caudal was the only vestibular neurongroup to span multiple rhombomeres (r4-r7), though it exhibited an internal cytoarchitecture wherein subcomponents of the group localized to individual rhombomeres [121].

Another study used grafts of quail neural tube inserted in varying positions within the isthmus and r1-r2 region of stage HH11 chicks to determine that the locus coeruleus neurons originate in an intermediate position in the alar plate of the r1 region. As development proceeds, it was noted that the locus coeruleus neurons in the alar plate migrate tangentally to a more ventral area close to the alar-basal plate boundary [122]. Thus, transplant procedures of the prospective rhombencephalon have allowed researchers to conclude that regions corresponding to hindbrain structures typically fall within boundaries of whole rhombomeres, though there are cases where regions of presumptive hindbrain structures migrate or spillover slightly into adjacent rhombomeres or are spread discontinuously across many rhombomeres.

Tissue transplantation during early embryonic development has also shed light on the origins of blood cells in hematopoiesis. Yolk sac transplants between chick and quail embryos has indicated that hematopoietic cells are of yolk sac origin, including a group of macrophage-like cells that partake in an early embryo phagocytic cell system of blood cell lineage, and endothelial cells are of intraembryonic origin [123, 124]. Reciprocal grafts of either the last somite (orthotopic) or the lateral plate mesoderm (heterotopic) to the last somite between quail and chick embryos has identified two distinct lineages in endothelial precursors: endothelial cells of paraxial mesoderm origin and of splanchnopleuric mesoderm origin. Of these two lineages, all hematopoietic cells were found to derive from the splanchnopleuric cells, and never from paraxial mesoderm cells [125]. In *Xenopus laevis*, orthotopic grafts of dorsal and ventral tissue has implicated that all hematopoietic precursor cells originate from the ventral mesoderm but are localized differently depending upon the age of the organism. Hematopoietic precursors of embryos localized in the ventral blood island, while those formed in late larval stages and adulthood were localized in the dorso-lateral plate [126-128]. Reciprocal transplants of dorsal lateral plate mesoderm in stage 14-15 *Rana pipiens* frogs has shown that subsequent to formation in the dorsal lateral plate, hematopoietic precursors migrate from the posterior end of the embryo and by stage 20 invade the circulatory network via the anterior pronephoses and the dorsal aorta [129, 130].

Despite the focus on mapping of neural ectoderm and its derivatives, transplantation studies have also been utilized in the fate mapping of a variety of tissues and structures. In *Xenopus* embryos, orthotopic grafts of the dorsal and ventral marginal zones has established that the dorsal marginal zone contributes to a small portion of the body, namely the notochord and the anterior wall of archenteron, and the ventral marginal zone contributes only to posterior regions of the body, including posterior lateral plate, endoderm and somites [131, 132]. The migration of epithelial surface cells to deep layers of the mesoderm to form somites, the origin of sensory placodes in the pre-placodal ectoderm, the fates of cells in the mouse epiblast to mesodermal derivatives, the contribution of the chick blastodisc in myocardium formation, and the formation of the anterior wall of archenteron from bottle cells have also been studied by transplantation methods [133-137]. Such research has facilitated a more comprehensive understanding of the movement and functions of different tissue systems across species and throughout development.

Analysis of gene function

It is clear from the studies described in previous sections that the use of molecular markers allowed precise assessment of the state of determination and competence of tissues as well as their inducing capacity. However this technique has also served as an invaluable and essential tool in delineating the role of genes in particular developmental processes. A specific method of transplants, termed mosaic analysis, involves transplanting a group of cells (typically 10-50 cells) from embryos with a mutant form of a gene to a wild-type embryo or vice versa and analyzing the behavior of the transplanted cells in the new environment. This can reveal whether a gene function is cell autonomous or cell non-autonomous, and elucidate in conjunction with additional markers the effect of the mutant gene on surrounding tissues. In this way, the contribution of specific genes within a developmental pathway can be observed separately, and then analyzed collectively. Mosaic analysis has most commonly been employed in genetically tractable models such as the zebrafish, as its robustness, accessibility, and relative transparency during embryogenesis facilitate the observation of interactions among individual cells. This method

has been employed in the study of a variety of different developmental processes that include mesendoderm specification, hematopoiesis, hindbrain development, and cell movements during gastrulation.

Mosaic analysis methods have been widely employed in the study of specific genes involved in hematopoiesis. Zebrafish *swirl* mutants, embryos that contain a mutated form of *bmp2b*, display expanded dorsal structures including notochord and somites, while more ventral structures including blood and nephros are absent. Cell transplants between wild-type zebrafish embryos and *swirl* mutants have implicated *bmp2b* as a non-autonomous contributor to blood cell specification, indicating that *bmp2b* acts within the BMP pathway as a ligand rather than a receptor or downstream signal transducer [138, 139]. The *smad5* (*sbn*) gene acts downstream of *bmp2b*, and mutants exhibit similar dorsalized phenotypes to the *swirl* mutants. Mosaic analysis of these mutants, termed *somitabun* mutants, has revealed that the action of *sbn* in the specification of ventral derivatives is non-autonomous. This suggests that the *bmp2/4* function during dorsoventral patterning is two-fold, with an initial *sbn*-independent phase and a later *sbn*-dependent phase, as *sbn* is required cell-autonomously for the downstream autoregulation of *bmp2b* in dorsoventral patterning before its non-autonomous role in the specification of blood cells and other ventral cell types [140].

Heterotopic transplants at the marginal zone between mutant and wild-type zebrafish for the *cloche* gene have revealed that the gene acts autonomously and non-autonomously during hematopoiesis. Transplants of wild-type cells to mutant hosts resulted in both wild-type and mutant cells expressing *gata1*, a genetic marker in red blood cells; however, wild-type cells were still far more likely to contribute to blood cells than mutant cells. This phenomenon suggests that *cloche* is required non-autonomously for expression of *gata1* during blood cell differentiation, but is required cell-autonomously in blood cells for their subsequent proliferation and survival following *gata1* induction [141]. Further mosaic analysis in *cloche* mutants in which transplants between wild-type and mutants also occurred at the marginal zone has indicated that in addition to its function in red blood cell development, *cloche* also acts in the generation of endocardial cells. Wild-type cells transplanted into mutant hosts are able to contribute to the endocardium, indicating that *cloche* function in endocardial cell differentiation is cell-autonomous and thus its gene product is likely to participate in a receptor signaling pathway [142]. Zebrafish *bloodless* gene, which putatively acts upstream of or in conjunction with the *cloche* gene, non-autonomously specifies hematopoietic progenitors *via* a surface receptor signaling pathway, which has been indicated by transplants between mutants and wild-type embryos; wild-type cells transplanted to the margin of mutant donors are unable to express *gata1*, while mutant cells transplanted to wild-type hosts can become specified into hematopoietic progenitors and express *gata1* [143]. Mosaic analysis of *spadetail* (*spt*) mutants in which embryonic red blood cells are absent in the growing embryo, has revealed that hematopoietic progenitors require interactions with the paraxial mesoderm for red blood cell differentiation; transplants between wild-type and mutants have shown that *spt* function is required autonomously within cells of the intermediate mesoderm as well as non-autonomously in paraxial mesoderm cells in order for red blood cell differentiation to take place [144].

The formation and specification of mesendodermal precursors and their derivatives has also been widely studied through the generation of mosaics by cell transplantation. Transplants of mutant cells to wild-type zebrafish embryos revealed that the *one-eyed pinhead* (*oep*) gene functions cell-autonomously in the specification of mesendodermal precursors, but cannot autonomously direct cells to internalize to the marginal deep layer in the same way seen in wild-type embryos alone. Similar mutant to wild-type and wild-type to mutant transplants have also implicated *oep* in the autonomous formation of the floor plate, prechordal plate, hatching gland, endoderm, and the axial mesoderm. It is presumed to act as an extracellular cofactor for the *cyclops* (*cyc*) and *squint* (*sqt*) genes [145-147]. Mosaic analysis of *cyc*, a key component in nodal signaling and mesoderm induction, has provided further insight into the formation of the floor plate. Transplants of neuroectoderm and mesoderm between mutants and wild-type zebrafish embryos have revealed both autonomous and non-autonomous roles, respectively, for *cyc* in the differentiation of

floor plate; this data has led to the conclusion that floor plate induction occurs along two pathways: induction from the notochord, which is regulated by autonomous expression of *cyc*, and homogenetic induction between floor plate precursors *via* non-autonomous action of *cyc* [148, 149].

Mutant to wild-type transplants of another key mesoderm-inducing factor *sqt* have shown that wild-type cells in *oep* mutant embryos (which lack nodal signalling function) can express the mesodermal marker *no tail* in response to distant sources of *sqt*. This indicates that *sqt* is a secreted morphogen that can act autonomously of nodal signaling, and thus the induction and patterning of the mesoderm involves both short- and long-range signaling mechanisms [150]. Cell transplants between wild-type and mutant zebrafish embryos of notochord-inducing genes *floating head* and *no tail* have indicated that these genes are required autonomously for notochord formation *via* the maintenance of notochord-inducing axial mesoderm and the differentiation of notochord precursors, respectively [151-153]. The generation of mosaic zebrafish embryos for both notochord (*ntl*, *flh*, and *doc*) and prechordal mesoderm (*cyc1* and *oep*)-inducing genes have additionally indicated that, contrary to previous beliefs, floor plate cells are induced largely by the prechordal and axial mesoderm rather than the notochord, which require non-autonomous expression of *neckless* for their own formation [147, 148, 153-155]. Transplants along the marginal zone between wild-type and zebrafish carrying the *casanova* (*cas*) mutation, a gene that functions in endodermal differentiation downstream of *cyc*, *sqt*, and *oep*, have indicated that *cas* functions cell autonomously within the endoderm to specify endodermal cell fate by activating endodermal markers like *sox17* independently of nodal signalling. Proper endoderm formation and differentiation, however, still requires sustained nodal signaling in addition to *casanova* expression [156-158]. Working in parallel to this pathway, the zebrafish *spg* gene works in cooperation with *cas* to induce *sox17* expression during endoderm differentiation; mosaic analysis has indicated that *spg* is required autonomously during the transition from mesendoderm precursors to endodermal precursors [159].

Research dedicated to the specification and differentiation of the rhombomeres during hindbrain development has extensively used cell transplant techniques to elucidate the functions and interactions of various hindbrain patterning genes. Zebrafish *spiel-ohne-grenzen* (*spg*) gene is widely expressed in both the neuroectoderm and the underlying mesoderm during early neural development. *Spg* mutants display disruptions in the *pou2* gene, which is expressed only in the neuroectoderm; neuroectodermal as well as mesodermal transplants between *spg* mutants and wild-type embryos has indicated that *spg* expression is required only in the neuroectoderm for proper expression of molecules involved in the midbrain-hindbrain boundary formation, and that this requirement is cell-autonomous: the mesodermal layer alone cannot support ectodermal expression of these markers [160]. Segmentation of the hindbrain has been shown to be regulated by the zebrafish *valentino* and *krox20* genes, which are activated upstream by vhnf1 and fgf signals [161-163]. Mosaic analysis of *fgf3/fgf8* through r4 transplants between mutant and wild-type embryos has shown that wild-type cells are required in r4 to express fgf signals in order to promote expression of *krox20* in the presumptive r5, a marker for r5 identity; transplants of *valentino* (*val*) mutant cells in *fgf3/fgf8* mutants result in the non-autonomous rescue of *krox20* expression, indicating that *val* acts downstream of *fgf3/fgf8* in the specification of r5 identity by *krox20* expression [162]. Mosaic analysis of *val* alone has shown that the gene is required in the formation of the r5-r6 boundary by inducing downstream expression of *ephB4a* as well as required cell-autonomously in the specification of r5 and r6 fates; transplants in the presumptive hindbrain between *val* mutants and *vhnf* mutants have indicated that this specification of differential neuronal identity between rhombomeres is separate from the specification of rhombomere-specific cell surface receptors in r5 and r6 [161, 163, 164].

Similar transplants have been performed for *kreisler*, the mouse homolog of *valentino*, which has also been shown to be required cell-autonomously in the specification of r5 and r6 fates. However, *kreisler*-mutant cells transplanted into wild-type hosts display the ability to mix freely with cells in r6 and not r5, while zebrafish *valentino* mutant cells cannot mix with either r5 or r6 when transplanted into a wild-type environment, indicating that major

differences exist in the evolution of rhombomere specification processes between species [161, 165, 166]. *Krox20* expression in itself has been shown, through mutant to wild-type rhombomere transplants in chick embryos, to be autonomously required in the r5 region, but requires interactions from neighboring rhombomeres for expression in r3 [167].

In addition to the major areas studied by mosaic analysis, cell transplants have also been employed to explore craniofacial development, eye development, cellular movements, and other phenomena. Mosaic analysis has implicated the zebrafish *lockjaw* gene non-autonomously in the formation of mesodermally-derived muscles, skeletogenesis, and melanophore development as well as autonomously in the migration and contribution of neural crest cells to the pharyngeal arches [168-170]. Both zebrafish *casanova* and *chinless* genes have also been implicated in pharyngeal arch development, although cell transplants have indicated that *cas* requirement for pharyngeal cartilage formation is environmental, while *chinless* exhibits both autonomous and non-autonomous roles in cartilage and muscle formation [171, 172]. During eye development, mosaic analysis in zebrafish has revealed a non-autonomous requirement for both masterblind and silberblick in the neuroectoderm for proper formation and separation of the eyes, respectively [171, 173]. Dorsal convergence of cells during gastrulation requires autonomous *spadetail*, *mission impossible*, and *trilobite* function in zebrafish, as indicated by the ability of wild-type cells to converge normally when transplanted into mutant hosts, while lateral divergence movements require non-autonomous function of *no tail* in midline tissues [174-177].

Gastrulation in mouse models has also been examined by mosaic analysis, which has implicated an autonomous role for nodal signaling in the ectoderm for the formation of the primitive streak [178]. Transplants along the marginal zone between zebrafish *oep* and wild-type embryos have shown that the anterior mesoderm, which requires proper *oep* expression for its formation, is necessary for cell movements such as convergence and internalization in the neural plate to occur [179]. Motor neuronal migration requires both autonomous and non-autonomous function of zebrafish *trilobite* in the surrounding hindbrain tissue, as demonstrated by the failure of most wild-type neurons to migrate normally out of rhombomeres r4, r6, and r7, while still a significant amount of others (33% of wild-type cells) are able to migrate normally [175]. Similarly, an analogous study performed in similar regions of zebrafish embryos has indicated that the zebrafish *lzr* gene also functions non-autonomously in surrounding tissues to control motor axon pathfinding [180]. Overall the utilization of transplantation methods to study single gene function has allowed for the elucidation of signaling source versus receptor-coding genes, the sources of induction of various developmental processes, and the interactions between genes that are necessary for proper development.

Future directions

It is clear from the experiments described in the preceding sections that embryonic tissue transplantation has had a major impact on the field of developmental biology beginning with the birth of experimental embryology and carrying through to discoveries that relied on the use of precise gene expression assays for identifying cell identity and unambiguous host and donor markers. Tissue transplantation has led to a clear delineation of the state of determination or commitment of each tissue and presumptive organ in a wide variety of organisms, and has enabled the identification of genes that confer this state of near irreversible commitment. Likewise this approach has defined the competence of tissues to respond to inducing signals, as well as the ability of specific tissues and cells to provide inducing signals. Additionally transplantation has allowed precise fate-mapping of groups of cells and even individual cells with impressive resolution throughout development and has been instrumental in investigating the molecular basis of plasticity, an emerging field relevant for regenerative medicine. Finally, the ability to transplant tissues and even single cells carrying a specific mutation into a wild type embryo or wild type cells into an embryo with a mutant background has provided key information on the functions of hundreds of key developmental genes, delineating whether a particular gene acts cell autonomously or not. However while transplantation experiments have been essential for past insights in developmental biology, with newer technologies and emerging areas of interest,

the use of tissue transplantation has the potential to keep providing new insights regarding embryonic development.

One area of interest that spans many fields across the life sciences is that of plasticity – the ability of entities ranging from a cell or cells to an entire organism to respond to and recover from perturbations in the environment. As evidenced from an entire issue of *Nature* being devoted to this topic [76], understanding the mechanisms underlying plasticity is not only important for basic science and understanding the processes of determination and differentiation, but for regenerative medicine. A host of clinical approaches rely on the transplantation of stem cells directed to differentiate into specific cell and tissue types into hosts with the goal of replacing or repairing damaged tissues. Understanding the fundamentals of host-donor interactions is imperative and many of the experiments described above could serve as excellent model systems for furthering our understanding of this process. Applying the power of RNA-Seq, particularly single cell RNA-Seq, to transplantation studies, could identify global responses to transplantation and identify key transcriptional networks that re-pattern both the donor and neighboring host cells. Excitingly, recent advances in RNA-Seq technology like tomo-seq make it possible to acquire transcriptional information in 3D [181], thus integrating gene expression profiles with embryonic patterns. This could possibly be used to tease out the difference in donor and host tissue transcriptomic response to transplantation with some modifications. The expression of key genes identified *via* RNA-Seq could be visualized in real time as the transplanted tissue and host adapt to the perturbation using transgenic approaches.

Understanding the extent and limits of plasticity of both host and donor tissues is essential for the field of regenerative medicine. However transplantation experiments are relevant for this growing branch of medicine for another reason, namely, the considerable effort to develop and transplant a wide variety of bioengineered structures, including hybrid artificial constructs and stem cell-derived organoids grown *in vitro* [108]. On one hand, since these structures have yet to achieve the level of sophistication seen in adult animals but closely resembles embryonic tissues, its transplantation into embryos of corresponding stages could allow the evaluation of the feasibility of such technique. On the other hand, such experiments would also allow researchers to probe the mechanisms by which the donor tissue establishes neural connections with the host central nervous system, as well as possible ways to enhance this ability [182]. In addition, combining the use of sequencing and transplantation for regenerative research will open up a new field in both basic developmental biology and biomedical science.

CONCLUSION

Transplantation during early embryonic stages is a classical technique in developmental biology that has significantly contributed to our understanding of a plethora of issues. Through a variety of transplantation schemes, scientists have made significant breakthrough in understanding the timeline of specification and determination, inductive ability and competence, as well as the eventual fate of diverse embryonic tissue during early embryonic development. Its use has spanned most common vertebrate model organisms, organ and tissue types, and developmental stages. Modern molecular techniques have allowed more accurate and mechanistic investigation of embryonic development using transplantation. In addition to novel uses such as single-gene analysis, these new techniques will allow for the reinvestigation and expansion of earlier transplant studies, thus paving the way for discoveries of greater detail and accuracy in the field. With the rapid advancement of global genomic and transcriptomic analysis methods such as RNA-Seq and differential gene expression analysis, it is now possible to comprehensively examine the molecular nature of embryonic responses to tissue transplantation. Such analysis should become the direction of future transplantation studies on early embryos, as it will further contribute to the transition of early embryonic transplantation studies from descriptive to the quest of underlying molecular mechanisms. Moreover, the integration of such techniques with regenerative medicine would have significant impacts on basic and clinical science.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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