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REVIEW ARTICLE

In vitro organogenesis using multipotent cells

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Abstract

The establishment of efficient methods for promoting stem cell differentiation into target cells is important not only in regenerative medicine, but also in drug discovery. In addition to embryonic stem (ES) cells and various somatic stem cells, such as mesenchymal stem cells derived from bone marrow, adipose tissue, and umbilical cord blood, a novel dedifferentiation technology that allows the generation of induced pluripotent stem (iPS) cells has been recently developed. Although an increasing number of stem cell populations are being described, there remains a lack of protocols for driving the differentiation of these cells. Regeneration of organs from stem cells *in vitro* requires precise blueprints for each differentiation step. To date, studies using various model organisms, such as zebrafish, *Xenopus laevis*, and gene-targeted mice, have uncovered several factors that are critical for the development of organs. We have been using *X. laevis*, the African clawed frog, which has developmental patterns similar to those seen in humans. Moreover, *Xenopus* embryos are excellent research tools for the development of differentiation protocols, since they are available in high numbers and are sufficiently large and robust for culturing after simple microsurgery. In addition, *Xenopus* eggs are fertilized externally, and all stages of the embryo are easily accessible, making it relatively easy to study the functions of individual gene products during organogenesis using microinjection into embryonic cells. In the present review, we provide examples of methods for *in vitro* organ formation that use undifferentiated *Xenopus* cells. We also describe the application of amphibian differentiation protocols to mammalian stem cells, so as to facilitate the development of efficient methodologies for *in vitro* differentiation.

Key words: animal cap, embryonic stem cell, organogenesis, Xenopus.

CARDIAC ORGAN FORMATION IN *XENOPUS LAEVIS*

During the early development of *Xenopus*, cardiogenesis is initiated at the early gastrula stage by a combination of signals from Wnt antagonists derived from the Spemann organizer and various endodermal factors.[1–3] Cardiac progenitor cells are formed bilaterally on mesodermal tissues that lie adjacent to the Spemann organizer. The presumptive cardiac mesoderm (PCM) migrates laterally toward the anterior surface as invagination proceeds, eventually fusing at the ventral midline in the tailbud stage. Subsequent differentiation of the cardiac tissue and formation of the beating heart occur at this ventral location (Fig. 1). Cloning and functional analyses of heart-specific genes are useful for elucidating the mechanism of heart differentiation. However, it is difficult to isolate

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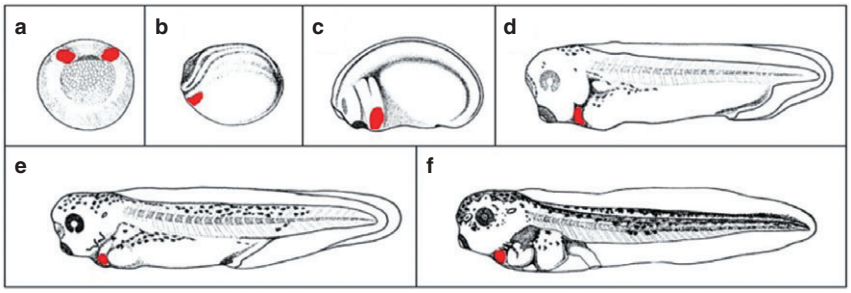

Figure 1 Cardiac formation during *Xenopus* early embryogenesis. (a) Precardiac mesoderm (PCM: red) is formed on the prospective dorsal side of the early gastrula (stage 10). (b) By the early neurula stage (stage 14), the PCM is fused and lies at the anterior end of the embryo. (c) At the early tailbud stage (stage 23), the PCM is separated laterally. (d) At the late tailbud stage (stage 34), a tubular and beating heart is formed. (e) At the late tailbud stage (stage 38), the heart separates into the atrium and ventricle. (f) At the tadpole stage (stage 42), dynamic heart looping occurs. (a) Vegetal view, with dorsal side upward. (b–f) Lateral view, with anterior side toward the left. Developmental stages are assigned according to Nieuwkoop and Faber.[4]

manually the endogenous PCM due to its small size (Fig. 1).

Previous studies have shown that explants of the non-cardiogenic ventral marginal zone (VMZ) form a beating structure through the overexpression of *Xwnt11*, *crescent* or *dkk1*.[3,5] In this differentiation system, transcripts of these genes are microinjected into the equatorial regions of the two ventral blastomeres of 4-cell stage embryos. These VMZ explants are isolated at the early gastrula and cultured. After 2 days, strong expression of the cardiac marker genes *Nkx2.5*, *Tbx5*, *TnIc*, and *MHC* is evident.[6–10] A few days later, the explants begin beating.

Pluripotent cells, which compose the "animal cap", are present in the blastula of *X. laevis*. This region, which comprises approximately 1000 cells, induces the differentiation of myocardial cells by activating activin[6,11] and inducing the overexpression of various proteins, including GATA4.[12] However, in all of the investigations conducted to date, the induction rate for myocardial cells was not high, and the structure of induced tissues did not resemble that of original heart.

Therefore, we established an experimental system in which beating hearts are induced with close to 100% probability, using a new procedure for temporarily dissociating cells from the animal cap with activin[13] (Fig. 2a). When these cells are cultured, rather than forming a mass of myocardial cells, they form a tubular structure. Whole-mount *in situ* hybridization of the *TnIc* gene, which is ordinarily expressed specifically in the myocardium of the heart, revealed that the beating tissues formed a heart-like structure (Fig. 2b). We then carried out an ectopic transplantation experiment, to determine whether explants form higher-order heart structures in the body and play some role in blood circulation (Fig. 2a). If ectopic hearts contribute to hemodynamics and form structures, such as atria and ventricles, this would represent a breakthrough in understanding cardiogenesis and organ engineering. When myocardial cells induced by this method were transplanted ectopically into the abdomens of other embryos, they contributed to systemic circulation in the same manner as the host heart (Fig. 2c). Moreover, secondary hearts possessed higher-order structures, such as atria and ventricles, and were morphologically, histologically (Fig. 2d), and electrophysiologically identical to the original hearts. However, unlike ES cells, these cells did not become tumorigenic. Although these results have no immediate clinical application, our finding that a heart induced *in vitro* can form higher-order structures in the body and functions as a circulatory organ represents a major advance in heart regeneration research.

The abovementioned *in vitro* heart differentiation systems should prove very useful for analyzing at the molecular level cardiogenesis in vertebrates. These systems enable rapid increases in the rate of cardiogenesis through the use of undifferentiated animal caps or VMZ as the starting materials, coupled with a simple procedure for one-time dissociation of the animal cap. This system should enable the identification of novel genes that are involved in the earliest stages of cardiogenesis, the analysis of which has proven difficult in previous experimental systems that used the presumptive cardiac region as source material. In addition, since *Xenopus* eggs are fertilized externally, all stages of embryo development are easily accessible, making it relatively easy to study the functions of individual gene products during heart development, using

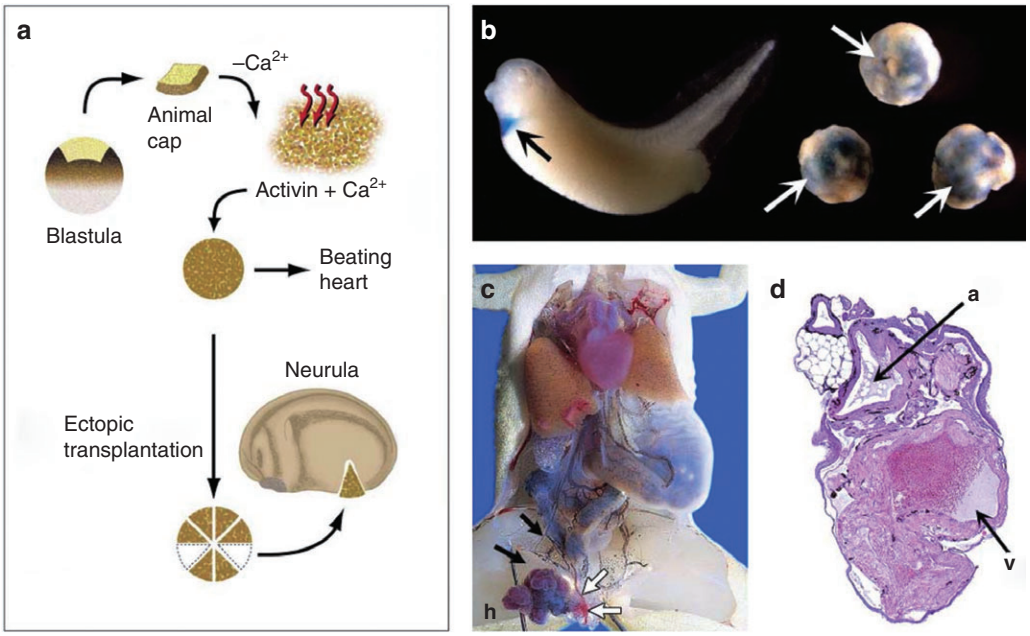

Figure 2 Cardiac induction from animal caps and *in vivo* transplantation. (a) Animal caps excised from the blastula were placed in calcium-free saline, to loosen the cell adhesions. The culture medium was replaced with 100 ng/mL activin dissolved in saline that contained calcium, and the cells were dissociated by pipetting. Individual cells were then reaggregated, and the reaggregates began to beat approximately 2 days later. Reaggregates that had been cultured for 1 day were cut into pieces of appropriate size and transplanted through an incision anterior to the cloaca in the early tailbud. (b) *TnIc*, which is a marker gene for myocardial differentiation, is expressed specifically in the myocardium of a normal 3-day-old embryo (left). The dissociated animal cap cells form spherical reaggregates, regardless of whether or not they have been treated with activin. *XcTnI* signals are detected exclusively in the activin-treated reaggregates (right). (c) Internal anatomy of a 1-year-old frog with a well-developed ectopic heart. The ectopic heart adjacent to the host's intestine is incorporated into the host's vascular system. The blood from the host's mesenteric artery (black arrows) flows into the host's anterior abdominal vein (white arrows) *via* the ectopic heart (h). (d) Histologic section of an ectopic heart. The heart can be divided into multiple chambers: a ventricle-like chamber (v), which comprises a thick and deeply penetrating layer of myocardium, and an atrium-like chamber (a), which is surrounded by a thin layer of myocardium.

microinjection into embryonic cells.[14–16] Blastomere-specific microinjection allows widely expressed genes to be disrupted in a specific way in differentiating heart tissues. Furthermore, since *Xenopus* heart function is entirely unnecessary until the tadpoles begin feeding, treatments that severely disrupt cardiogenesis can be studied in this model.[17–19]

We originally set out to investigate the genes involved in cardiogenesis. Subsequently, we cloned the *Xenopus hyaluronan* and *proteoglycan-binding link protein 3* (*XHAPLN3*) genes, which ar expressed in the cardiac region during early embryogenesis.[17] HAPLN family proteins contain an aggrecan family-binding domain and two hyaluronan-binding domains. The *XHAPLN3* mRNA transcripts overlapped with the *Xhas2*[20,21] and *Xversican* transcripts in the heart anlage of early tailbud embryos (Fig. 3a–d). Furthermore, knockdown of *XHAPLN3* or *Xhas2* with specific morpholino antisense oligonucleotides resulted in heart deficiency in developing tadpoles (Fig. 3e–g). Further analysis indicated that XHAPLN3 helps to maintain the hyaluronan matrix around the cardiac anlage, thereby contributing to cardiogenesis. Therefore, the screening of *in vitro*-induced hearts contributes information as to when and how components of the hyaluronan matrix function in cardiogenesis.

We then explored a comprehensive set of cardiogenesis-related genes using the *Xenopus in vitro* system and microarray. This screening has identified several cardiogenesis-related genes, promising new findings regarding heart formation. We aim to exploit the knowledge gained from these studies to elucidate the

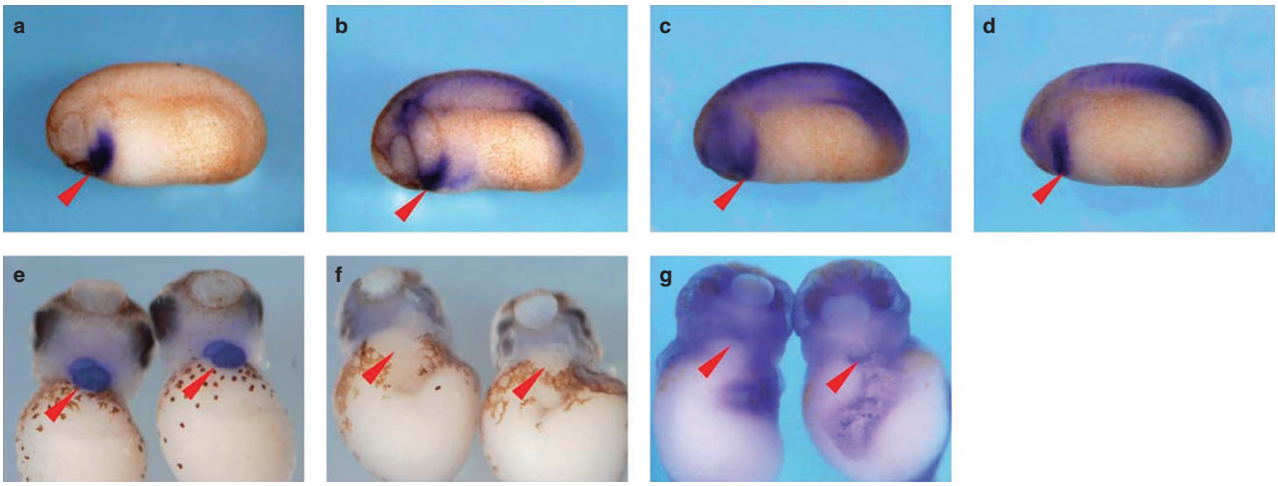

Figure 3 Developmental expression profiles and knockdown analyses of *XHAPLN3* and *Xhas2*. (a–d) Whole-mount *in situ* hybridization of *Nkx2.5* (a), *XHAPLN3* (b), *Xversican* (c), and *Xhas2* (d). Lateral views are shown, with the anterior side towards the left and the dorsal side upward. The arrowhead indicates the precardiac mesoderm (PCM). (e–g) The effects of depleting XHAPLN3 and Xhas2 are validated by the pattern of *TnIc* expression. Normal tadpole heart structure (e: arrow). Neither the XHAPLN3-morphant (f) nor the Xhas2-morphant (g) show *TnIc* expression. Ventral views are shown, with the anterior side upward.

mechanisms of organ formation, to create an organ formation "roadmap," and to advance organ differentiation and regeneration technologies (Fig. 4).

INDUCTION OF VASCULAR ENDOTHELIAL CELLS FROM UNDIFFERENTIATED *XENOPUS* CELLS

The origin of vascular endothelial cells has long been regarded as being linked to that of hematopoietic cells.[31] Several reports have suggested the existence of bipotential progenitor cells, that is, hemangioblasts, which can differentiate into both vascular endothelial cells and blood cells. The existence of hemangioblasts is supported by the finding that the loss of function or forced expression of several genes affects both cell types. For example, mouse embryos that are homozygous for *Flk-1* lack both vascular structures and expression of the hematopoietic marker Gata-1.[32] Overexpression of the hematopoietic transcription factor Scl/tal-1 in combination with the LIM domain transcription factor Lmo2 induces the expansion of *Gata-1* expression and *Fli-1* expression in zebrafish.[33] Despite substantial indirect evidence, hemangioblasts have not been isolated *in vivo*, and the mechanisms underlying the initial specification and subsequent separation of vascular endothelial and hematopoietic precursors remain unknown. In the first stage of blood vessel development, vascular endothelial cells are differentiated from hemangioblasts and form a vascular structure, concomitant with the formation of the main blood vessels (Fig. 5). This process is termed "vasculogenesis". In the second stage, which is called "angiogenesis", new vascular structures sprout from preexisting blood vessels, elongate, and become interconnected, so that a network of blood vessels is generated.

It has been previously reported that treatment with low concentrations of activin promotes the differentiation of ventral mesodermal cells, including blood cells, coelomic epithelial cells, and mesenchymal cells, in the explants of animal caps.[34,35] We assume that these blood cells are differentiated from hemangioblasts in the activin-treated explants. To define the optimal conditions for the differentiation of only vascular endothelial cells, we treated dissociated cells from animal caps with 0.4 ng/mL activin and 100 ng/mL angiopoietin-2 (Ang-2) for 1 h, and then allowed reaggregation;[36] this treatment resulted in the expression of the vascular endothelial markers *X-msr* and *Xtie2*[37,38] (Fig. 6), whereas there was no expression of the hematopoietic marker GATA-1,[39] in the reaggregated explants.[36]

Using this differentiation system, we carried out microarray analyses to compare the gene expression profiles of activin-treated and activin + Ang-2-treated animal cap cells, and identified vascular-specific genes, for example, the gene for XRASGRP2.[40,41] Ras guanyl

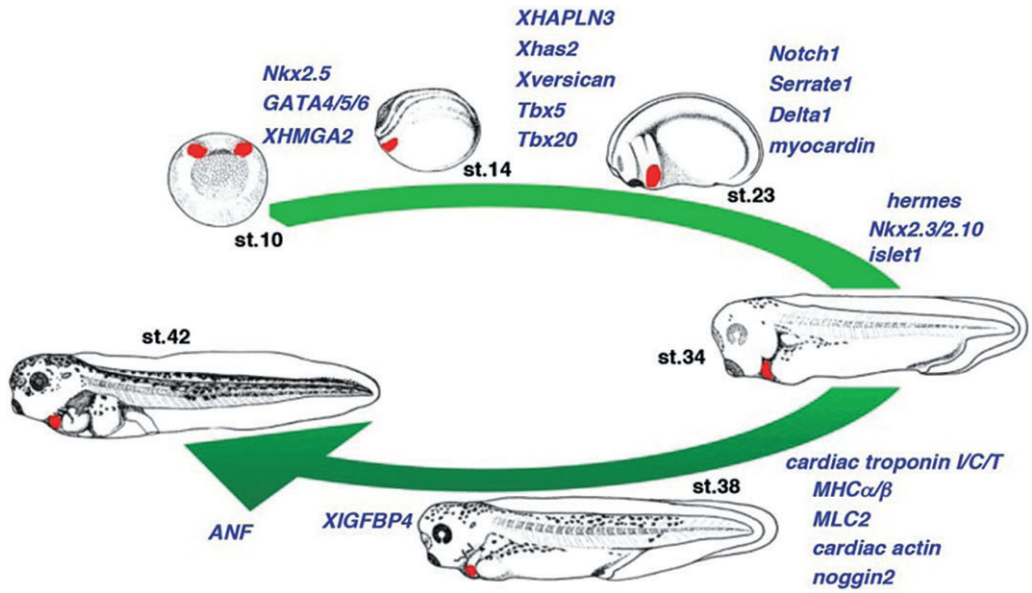

Figure 4 Already known genes identified using the microarray. Gene names are indicated in blue and arranged according to the embryonic stage. *Nkx2.5* and *GATA4/5/6* are among the earliest heart-related transcription factors.[8,22]Moreover, *Nkx2.5* expression is regulated by both *XHMGA2* and *Smad*s.[18]The RNA-binding protein *hermes* also regulates *Nkx2.5* expression.[23]*Tbx5*, *Tbx20*, *myocardin*, and *islet1* are transcription factors that interact with *Nkx2.5* and *GATA*s and regulate gene expression in the precardiac region.[24–26]Both the Notch signal (*Notch1*, *Serrate1*, and *Delta1*) and hyaluronan matrix (*Xhas2*, *Xversican*, and *XHAPLN3*) genes are essential for appropriate specification of myocardial cell fate.[17,27]Terminal differentiation marker genes (*cardiac troponin I/C/T, MHCa/b, MLC2, cardiac actin*, and *ANF*), which principally encode structural proteins, are expressed.[7,28,29]Moreover, cardiogenesis is maintained by several secreted factors.[19,30]

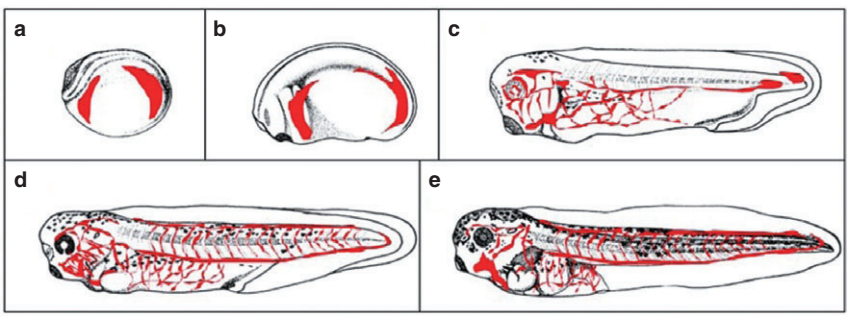

Figure 5 Vasculogenesis during early embryogenesis in *Xenopus*. (a) Precardiac mesoderm (PCM; red) is formed on the prospective dorsal side of the early gastrula (stage 10). (b–g) In the first stage of blood vessel development (vasculogenesis), vascular endothelial cells are differentiated from the hemangioblast and form a vascular structure, with the main blood vessels being formed (Fig. 5). In the second stage (angiogenesis), new vascular structures sprout from pre-existing blood vessels, elongate, and become interconnected, so that a network of blood vessels is generated.

nucleotide-releasing protein 2 (RASGRP2), which is one of the Ras guanine exchange factors, is implicated as a critical regulator of inside-out integrin activation in human lymphocytes, neutrophils, and platelets. XRASGRP2 overexpression induced ectopic vascular formation, and XRASGRP2-knockdown embryos showed delayed vascular development (Fig. 7). These results suggest that XRASGRP2 is essential for vascular formation during *Xenopus* development. As mentioned above, improvements to this method of *in vitro* induction are

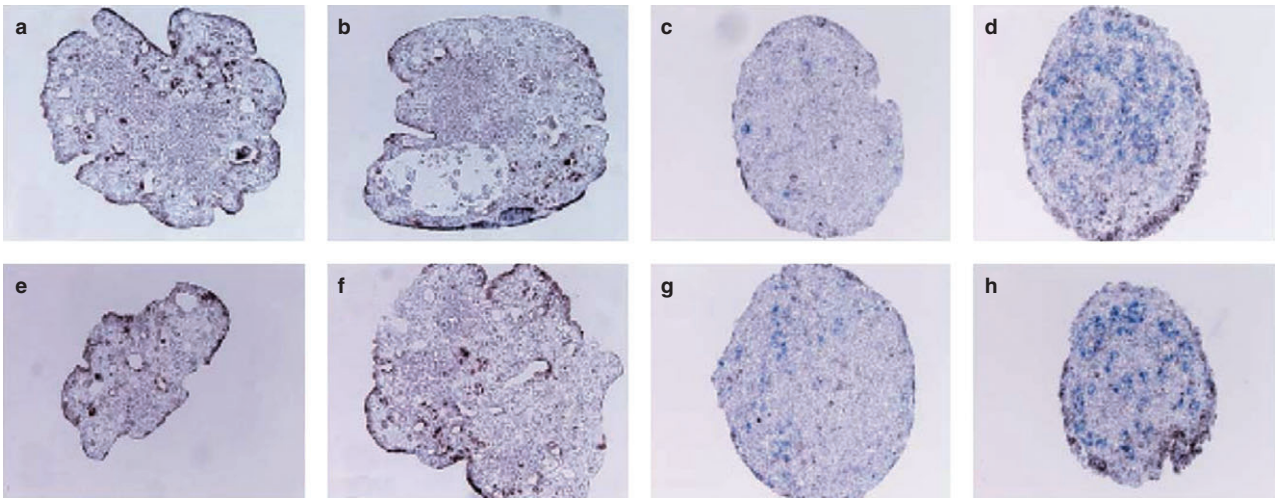

Figure 6 Expression of Xmsr and Xtie2 in aggregates. Sections of aggregates were hybridized with vascular-specific marker genes. (a–d) Xmsr; (e–h) Xtie2. (a,e) No treatment; (b,f) treatment with 100 ng/mL Ang-2; (c,g) treatment with 0.4 ng/mL activin; (d,h) treatment with 100 ng/mL Ang-2 and 0.4 ng/mL activin.

expected to facilitate the identification of many angiogenesis-related genes, as well as the development of a vascular endothelial "roadmap" (Fig. 8).

IN VITRO DIFFERENTIATION OF PRONEPHROS

The kidney, which is one of the excretory organs that is differentiated from the intermediate mesoderm, has three distinct morphologies, the pronephros, mesonephros, and metanephros, which undergo both evolutionary and developmental changes during organogenesis. These three forms express the key functions of the excretory system and the fundamental structure, called the nephron; the numbers of nephrons in these three renal organs differ. In frogs, the pronephros differentiates at 3 days postfertilization and functions during embryogenesis, and it is replaced by the mesonephros at adult stage 4. *In vitro*-induced kidney tissues from *Xenopus* animal caps are in the pronephros form. The first report of *in vitro* induction of pronephros appeared in 1993.[44] Animal caps isolated from late blastulas were treated simultaneously with a moderate dose of activin (1–10 ng/mL) and a high dose of retinoic acid (10^{-5}–10^{-4} M), and cultured in amphibian saline for 4 days. The induced pronephros contains the nephron structure, which consists of a tubule, duct, and glomus.[44–46] Gene expression in the induced pronephros was equivalent to that in the normal embryonic pronephros.[47] Furthermore, we carried out transplantation experiments to examine the functions of the *in vitro*-induced pronephros.[48] Surgical resection of the anlagen of the pronephric tubules caused disruption of pronephric development in frog embryos. These tadpoles presented with edema, and the embryos could be restored by transplantation of a single animal cap explant treated with activin and retinoic acid.[48]

Although the induction mechanism remains unclear, it is clear that activin induces the mesoderm in animal caps and that retinoic acid then modifies the mesoderm to generate the pronephric anlage. The *in vitro* induction system is applicable to ES cells; it has been reported that ES cells treated with activin and retinoic acid differentiate into nephric tissues.[49,50] We also applied the system to elucidate the molecular mechanism of pronephros development. By comparing the gene expression patterns of animal cap explants prepared for differentiation into pronephros and other tissues, we discovered that the *spalt* gene family is involved in vertebrate kidney development.[51,52] *Sall1*, which is a member of the *spalt* gene family, is not only essential for murine kidney development, but is also responsible for Townes-Blocks syndrome with renal anomalies.[53] These findings indicate that the *in vitro* induction system in *Xenopus* contributes not only to the establishment of stem cell engineering, but also to the discovery of novel factors that are essential for normal development in vertebrates.

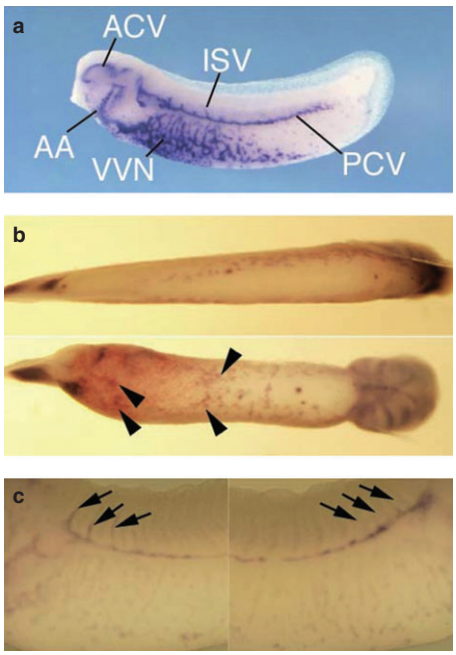

Figure 7 XRASGRP2 is essential for vascular formation during *Xenopus* development. (a) Endogenous expression of XRASGRP2. *XRASGRP2* transcripts are localized to the anterior cardinal vein (ACV), aortic arch (AA), intersomitic veins (ISV), posterior cardinal veins (PCV), and vascular vitelline network (VVN) at stage 30. (b) Whole-mount *in situ* hybridization for the endothelial marker *Xmsr* at stage 31; upper panel, expression of *Xmsr* in an non-injected control embryo (ventral view); lower panel, expression of *Xmsr* in an embryo that was injected into the VV with *XRASGRP2* and *β-gal*. Ectopic expression of *Xmsr* is evident at the injection site in the ventral blood island (VBI). Arrowheads indicate *Xmsr*-positive cells. (c) *XRASGRP2*-knockdown embryos generated by the injection of XRASGRP2-MO. The expression level of *Xmsr* in the ISV (left, black arrows) is diminished at the MO-injected side (right, black arrows).

IN VITRO PANCREAS INDUCTION

In vertebrate embryogenesis, the dorsal and ventral pancreatic buds are differentiated separately from the anterior endoderm and fuse before developing into a pancreas. *In vitro* induction of pancreatic tissue from *Xenopus* undifferentiated cells was established in 2000.[54] Whether the artificially induced pancreas bud in this system corresponds to the dorsal and/or the ventral pancreas bud remains unclear. Controlled time-lag treatment with a relatively high dosage of activin (100 ng/mL) plus a high dosage of retinoic acid (10^{-4} M) drives animal cap cells to differentiate into pancreatic cells in 10 days. The optimal time interval between activin and retinoic acid treatment is 3–5 h, and pancreas-like structures differentiate in 80% or more of the explants under these conditions. The probability of pancreas formation was found to be low for an interval longer than 5 h or for simultaneous treatment with activin and retinoic acid.[54] Under electron microscopy, the induced pancreas was observed to have the acinus-like structure for exocrine secretion, as well as secretory granules for endocrine function, and it was found to produce both insulin and glucagon.[54] The time interval between activin and retinoic acid treatment is crucial to controlling the induction system. A shorter time interval causes the animal caps to differentiate into pronephros rather than pancreas, while a time interval longer than 5 h decreases the probability of pronephros differentiation.[54,55] These studies suggest that regulation of the time interval of treatment with multiple inductive factors is a valuable approach for *in vitro* organogenesis.

The mechanism underlying the *in vitro* pancreas induction system is hypothesized to include the following steps: (i) a relatively high dose of activin initially induces the dorsal endomesoderm; and (ii) subsequent retinoic acid signaling modifies these cells to form the posterior pancreas. This hypothesis is supported by the fact that treatment of the isolated dorsal lip of the *Xenopus* early gastrula with retinoic acid induces pancreatic tissues.[56] Recently, similar induction systems that use activin and retinoic acid have been reported to enable the differentiation of murine ES cells into pancreas-like tissues that contain endocrine cells, exocrine cells, and duct-like structures,[57–60] details of which are given in the following ES cell section. Therefore, research on *in vitro* induction systems in *Xenopus* promotes stem cell engineering studies for regenerative therapy.

IN VITRO DIFFERENTIATION OF PANCREATIC CELLS FROM MURINE ES CELLS

Mammalian ES cells can be established from the inner cell mass of preimplantation embryos, and these cells are capable of unlimited self-renewal.[61,62] In contrast, animal cap cells are undifferentiated cells at the top of the animal pole area that can be transdifferentiated to a mesodermal fate by activin/nodal signaling. Interestingly, murine ES cells and *Xenopus* animal cap cells share some characteristics. For example, both cells have multipotent differentiation abilities towards various tissues

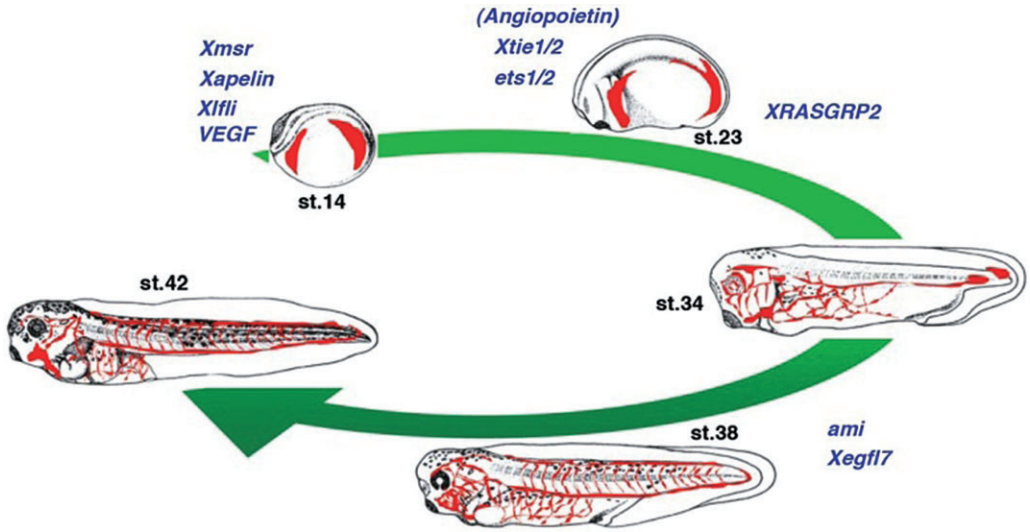

Figure 8 Already known genes identified using the microarray. Gene names are indicated in blue and arranged according to the embryonic stage. *Xlfli1* is a marker for hemangioblasts.[42]The start-points of vasculogenesis and angiogenesis are evidenced by the expression of *Xmsr* and *Xtie2*, respectively. Two novel potential marker genes, *ami*[43]and *XRASGRP2*, for the late differentiation of blood vessels, angiogenesis, and/or maturation, are identified.

in vitro, and they tend to differentiate spontaneously towards ectodermal lineages in the absence of external signals. These observations prompted us to hypothesize that amphibian differentiation protocols can be applied to the differentiation of mammalian ES cells.

First, we tested a method for the differentiation of ES cells into pancreatic tissues. As described above, the treatment of animal cap cells with a combination of activin and retinoic acid induced differentiation into pancreatic tissues. For the differentiation of murine ES cells, ES colonies grown on mouse embryonic fibroblast feeder cells were harvested by mild treatment with collagenase and dispase, and then grown in a suspension culture without serum for 4 days, so as to form embryoid bodies (EBs). The EBs were treated with a combination of activin and retinoic acid for 2 days, followed by adhesion culturing for 2 weeks without serum. As a result, in addition to a gut-like structure and a pancreatic duct-like structure, black spot-containing exocrine cells were efficiently differentiated from the EBs.[60] Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the induced pancreatic tissues expressed insulin, glucagons, somatostatin, pancreatic polypeptide, and amylase. Immunofluorescence staining of the differentiated cells revealed that they were positive for the exocrine cell marker amylase and the endocrine cell marker insulin C-peptide (Fig. 9). Furthermore, electron microscopy revealed that these

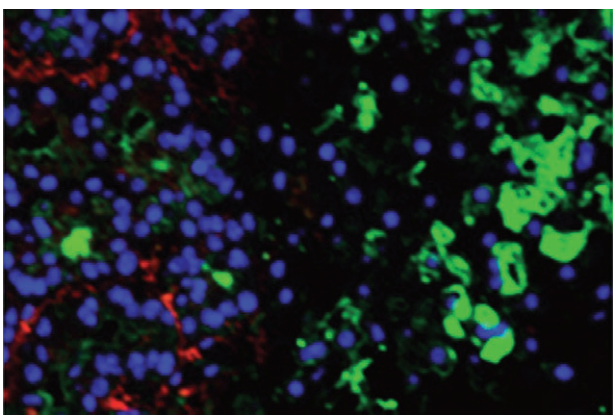

Figure 9 Pancreatic cell differentiation from murine embryonic stem (ES) cells. Embryoid bodies treated with activin and retinoic acid were cultured for 2 weeks on a gelatin-coated dish. The fixed cells were subjected to immunofluorescence analysis. Color code for staining: red, α-amylase; green, insulin C-peptide; blue: 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI).

cells had features characteristic of pancreatic acinar cells, that is, numerous zymogen granules and a well-developed endoplasmic reticulum. Cells that resembled pancreatic endocrine β-cells, which contain numerous endocrine granules, were also observed. Thus, these ES cells appeared to be terminally differentiated into pancreatic cells. We also found that the amylase-positive

exocrine cells were selectively induced at a lower concentration of activin (10 ng/mL), whereas treatment with a higher concentration of activin (25 ng/mL) specifically induced an enriched population of insulin-positive β-cells from the ES cells. This suggests that the concentration of activin significantly modulates the fate of the differentiating cell, as described for the amphibian animal cap model. Currently, we are developing a novel methodology for the differentiation of pancreatic cells using monolayer culturing and a chemically defined serum-free medium, so as to ensure the uniform differentiation of ES cells for regenerative medicine using stem cells.[63]

IN VITRO DIFFERENTIATION OF CARDIOMYOCYTES FROM MURINE ES CELLS

In the case of heart induction from murine ES cells, activin was not effective. However, a closely related growth factor, bone morphogenetic protein (BMP), efficiently induced cardiomyocyte differentiation.[64] Completely dissociated and thoroughly washed murine ES cells were aggregated in the presence of BMP4 when incubated without serum for 3 days, followed by adhesion culturing without BMP. In the absence of BMP4, the EBs spontaneously differentiated into neuronal lineages. In contrast, the addition of a low concentration of BMP (1 ng/mL) in serum-free medium switched differentiation from a neural to a mesodermal fate. Under these conditions, an early mesodermal marker, *brachyury*, and *goosecoid* were transiently induced. The lateral plate mesodermal marker *flk1* and the paraxial mesodermal markers *pdgfrα* and *pdgfrβ* were sequentially induced. Finally, after 9 days of culture, beating cardiomyocytes were observed. These cardiomyocytes were immunopositive for cardiac troponin T (Fig. 10), indicating that they were terminally differentiated. During the optimization of differentiation conditions, we noticed that the cell surface marker N-cadherin was significantly induced upon the initiation of cardiomyocyte differentiation. This finding prompted us to test whether cardiomyogenic precursor cells could be concentrated using an N-cadherin-specific antibody. As expected, the N-cadherin-positive fraction of BMP4-primed EBs showed a significantly strong ability to differentiate towards cardiomyocytes. These results suggest that N-cadherin is a useful cell surface marker for the efficient purification of cardiomyogenic precursor cells during stem cell differentiation. We are currently testing whether N-cadherin can be used as a prospective marker to segregate cardiomyogenic precursor cells from somatic stem cells.

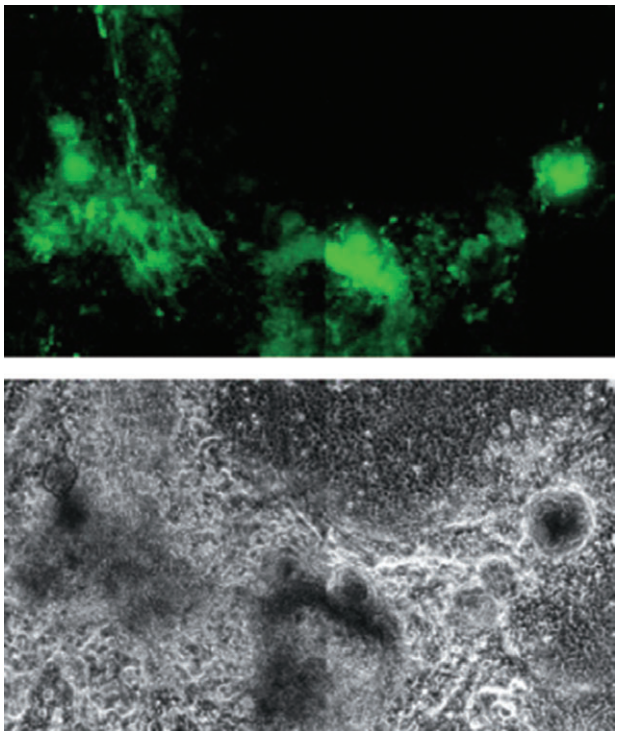

Figure 10 Cardiomyocyte differentiation from murine embryonic stem (ES) cells. Embryoid bodies treated with 1 ng/mL BMP4 were cultured for 9 days on a gelatin-coated dish. The fixed cells were subjected to immunofluorescence analysis. Upper panel: green, cardiac troponin T; lower panel: phase contrast microscopy.

IN VITRO DIFFERENTIATION OF EPITHELIAL CILIATED CELLS FROM MURINE ES CELLS

We have also established conditions for the differentiation of ciliated cells of respiratory tissues.[65] Completely dissociated murine ES cells were aggregated in the presence of serum for 3 days in round-bottomed plates, followed by adhesion culturing in serum-free medium without the addition of any growth factors. After 4 weeks, cells with cilium-like motility were readily observed. These cilium-like cells localized to the area of expanded outgrowth from almost all of the EBs cultured on a gelatin-coated dish. The cilium-like structure was immunopositive for β-tubulin IV (Fig. 11) and Foxj1,

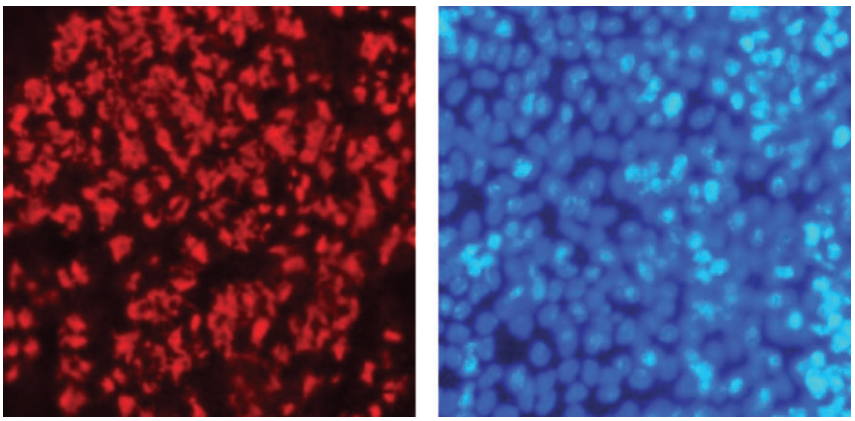

Figure 11 Ciliated cell differentiation from murine embryonic stem (ES) cells. Embryoid bodies were cultured without exogenous addition of growth factors for 28 days on a gelatin-coated dish. The fixed cells were subjected to immunofluorescence analysis. Color code for staining: red, b-tubulin IV; blue, 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI).

and under electron microscopy, it exhibited the 9 + 2 arrangement of microtubules that is typical of cilia, having axial filaments in the center of each cilium. These ciliated cells were positive for the lung markers *TTF-2* and *SP-C* and negative for the oviduct marker *ovgp1*, suggesting that they were ciliated cells from the trachea. The cilia of these cells beat at a frequency of 17–20 Hz, which is similar to the speed of endogenous cilia analyzed with a high-speed camera. These results indicate that functional ciliated cells are successfully induced under these conditions. Previous methods for inducing ciliated cells *in vitro* from embryonic or adult tissues required special differentiation conditions of culturing at an air-liquid interface. Our system more closely mimics the normal development of ciliated cells.

When various growth factors were tested with respect to the differentiation of ciliated cells, several BMPs were identified as strong inhibitors of differentiation. BMP2, BMP4, and BMP7 inhibited the expression of *FoxJ1* and induced the expression of the clara cell marker gene *CC10* and the mucus cell marker gene *MUC5AC*. BMP4 has been suggested to be important in determining the distal-proximal polarity of respiratory cells.[66] Our results suggest that the differentiation of ciliated cells from murine ES cells is driven by a similar mechanism: cells that express high levels of BMPs differentiate into the distal cells of the respiratory tract, whereas cells with low levels of expression of BMPs differentiate into proximal cells, including ciliated cells.

Based on these findings, we tested whether intracellular inhibitors of BMP signals, such as Smad6 and Smad7, promoted ciliogenesis from murine ES cells. The inhibitory Smads promoted the differentiation of ciliated cells even when the BMPs were incubated in a high concentration of serum. Moreover, the differentiated ciliated cells appeared to be from the brain, as they were positive for the ependymal marker musashi1. These results suggest that the inhibition of BMP signaling is crucial not only for ciliogenesis, but also for the subtype determination of ciliated cells that differentiate from ES cells.

IMPORTANT ISSUES REGARDING THE APPLICATION OF ES CELLS AND IPS CELLS TO REGENERATIVE MEDICINE

Embryonic stem cells are capable of unlimited symmetrical self-renewal and have the potential to differentiate into all the cell types found in adult tissues, including germ cells. Given these unique properties, ES cells are expected to have immense therapeutic potential in regenerative medicine. Although mesenchymal stem cells from adult bone marrow or fat tissues show multiple differentiation abilities towards bone, cartilage, and fat, and are now used in certain clinical applications, their differentiation abilities appear to be limited compared with those of ES cells. For example, mesenchymal stem cells do not readily differentiate into neurons. To overcome these limitations, ES cells are expected to be used in medical applications, despite the ethical issues involved. Recently, an American venture company, Jeron, initiated a clinical study using human ES cells for treating spinal cord injuries. In 2007, it was demonstrated that human adult tissue cells could be dedifferentiated into ES-like pluripotent stem cells (iPS cells) by introducing the defined transcription factors.[67,68] Although clinical applications require a more comprehensive characterization of iPS cells and the further development of various technologies for the generation and manipulation of iPS cells, this tailor-made ES-like cell has strong potential in the field of regenerative

medicine. Moreover, this artificially induced stem cell may be a good model for the dedifferentiation or transdifferentiation of adult tissue cells into the various cells required for regenerative medicine.

However, certain problems associated with the use of ES cells and iPS cells need to be resolved before their clinical application. Given the potent abilities of ES cells to differentiate into several cell types, it is clear that technologies to control differentiation are lacking. Therefore, further studies on the regulation of stem cell differentiation *in vitro* and *in vivo* are warranted. Moreover, these ES cells spontaneously generate teratomas when transplanted into nude mice, owing to their unlimited self-renewal and pluripotent differentiating abilities. Moreover, in the case of iPS cells, genomic incorporation of the four genes could theoretically disrupt endogenous genes essential not only for maintenance of the homeostasis of the differentiated cells, but also for the inhibition of tumor formation *in vivo*. Thus, contamination of the differentiated cells used for transplantation with even a low number of undifferentiated ES cells or iPS cells could result in tumor formation rather than successful cell therapy.

IDENTIFICATION OF PLURIPOTENCY-SPECIFIC CELL SURFACE MARKERS IN MURINE ES CELLS

To avoid failure of cell therapy, we attempted to identify cell surface markers that could be used to separate undifferentiated ES cells from differentiated cells. To identify membrane markers that are specifically expressed in undifferentiated ES cells, we carried out proteomic analysis of the cell surface membrane fractions of murine ES cells using two-dimensional high-performance liquid chromatography (2D-LC), followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Instead of the widely used strong cation exchange (SCX) chromatography, we applied zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) as the first-dimensional HPLC. ZIC-HILIC gives better fractionation than the traditional SCX chromatography.[69] Although SCX chromatography has a higher capacity for proteins, its fractionation efficiency is lower than that of ZIC-HILIC, and the elution curve of every peptide peak in SCX chromatography is broader than the corresponding peak in ZIC-HILIC[69]. We also used isobaric tags for relative and absolute quantitation (iTRAQ), to quantify the cell surface marker proteins expressed by undifferentiated ES cells and differentiated cells. As a result, we identified 300 membrane proteins that were expressed in undifferentiated and differentiated murine ES cells. Among these, approximately 40 proteins were expressed specifically in undifferentiated ES cells.[70] The identified membrane proteins included channel proteins, adhesion molecules, and receptors. These proteins may represent candidate cell surface markers that can be used to discriminate undifferentiated ES cells from differentiated cells for cell-based therapies. During the differentiation of ES cells *in vitro*, various growth factors and components of the extracellular matrix regulate the pluripotency or the differentiation of ES cell progenies. Therefore, these cell surface proteins are also expected to play key roles in the maintenance of pluripotency and in the regulation of ES cell differentiation.

CONCLUSION

In this review, we describe unique approaches to *in vitro* organ, tissue, and cell differentiation. We use *Xenopus* embryos and undifferentiated stem cells in a model system. Detailed analyses of organ formation during *Xenopus* embryogenesis generate blueprints that describe genes crucial for the differentiation of organs. Moreover, using animal cap cells, we have developed various *in vitro* organ differentiation protocols. These protocols can be applied to mammalian stem cells, including ES cells and iPS cells. We are also developing methods to avoid tumorigenesis associated with cell therapy by selectively using differentiated ES cells or iPS cells. These basic studies could contribute to the development of new technologies for regenerative medicine and reliable assay systems for drug discovery.

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REFERENCES

1 Nascone N, Mercola M. An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* 1995; **121**: 515–23.
2 Shi Y, Katsev S, Cai C, Evans S. BMP signaling is required for heart formation in vertebrates. *Dev Biol* 2000; **224**: 226–37.

3 Schneider VA, Mercola M. Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. *Genes Dev* 2001; **15**: 304–15.
4 Nieuwkoop PD, Faber J. *Normal Table of Xenopus Laevis (Daudin)*.New York: Garland Publishing Inc., 1994.
5 Pandur P, Lasche M, Eisenberg LM, Kuhl M. *Wnt-11* activation of a non-canonical *Wnt* signalling pathway is required for cardiogenesis. *Nature* 2002; **418**: 636–41.
6 Logan M, Mohun T. Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. *Development* 1993; **118**: 865–75.
7 Drysdale TA, Tonissen KF, Patterson KD, Crawford MJ, Krieg PA. Cardiac troponin I is a heart-specific marker in the *Xenopus* embryo: expression during abnormal heart morphogenesis. *Dev Biol* 1994; **165**: 432–41.
8 Tonissen KF, Drysdale TA, Lints TJ, Harvey RP, Krieg PA. XNkx-2.5, a *Xenopus* gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. *Dev Biol* 1994; **162**: 325–8.
9 Newman CS, Krieg PA. *tinman*-related genes expressed during heart development in *Xenopus*. *Dev Gen* 1998; **22**: 230–8.
10 Horb ME, Thomsen GH. Tbx5 is essential for heart development. *Development* 1999; **126**: 1739–51.
11 Ariizumi T, Komazaki S, Asashima M, Malacinski GM. Activin treated urodele ectoderm: a model experimental system for cardiogenesis. *Int J Dev Biol* 1996; **40**: 715–18.
12 Latinkic BV, Kotecha S, Mohun TJ. Induction of cardiomyocytes by GATA4 in *Xenopus* ectodermal explants. *Development* 2003; **130**: 3865–76.
13 Ariizumi T, Kinoshita M, Yokota C *et al*. Amphibian *in vitro* heart induction: a simple and reliable model for the study of vertebrate cardiac development. *Int J Dev Biol* 2003; **47**: 405–10.
14 Jacobson AG, Sater AK. Features of embryonic induction. *Development* 1988; **104**: 341–59.
15 Lohr JL, Yost HJ. Vertebrate model systems in the study of early heart development: *xenopus* and zebrafish. *Am J Med Genet* 2000; **97**: 248–57.
16 Mohun T, Orford R, Shang C. The origins of cardiac tissue in the amphibian, *Xenopus laevis*. *Trends Cardiovasc Med* 2003; **13**: 244–8.
17 Ito Y, Seno S, Nakamura H, Fukui A, Asashima M. XHAPLN3 plays a key role in cardiogenesis by maintaining the hyaluronan matrix around heart anlage. *Dev Biol* 2008; **319**: 34–45.
18 Monzen K, Ito Y, Naito AT *et al*. A crucial role of a high mobility group protein HMGA2 in cardiogenesis. *Nat Cell Biol* 2008; **10**: 567–74.
19 Zhu W, Shiojima I, Ito Y *et al*. IGFBP4-mediated Wnt inhibition is required for cardiomyogenesis. *Nature* 2008; **454**: 345–9.
20 Köprunner M, Müllegger J, Lepperdinger G. Synthesis of hyaluronan of distinctly different chain length is regulated by differential expression of Xhas1 and 2 during early development of *Xenopus laevis*. *Mech Dev* 2000; **90**: 275–8.
21 Nardini M, Ori M, Vigetti D, Gornati R, Nardi I, Perris R. Regulated gene expression of hyaluronan synthases during *Xenopus laevis* development. *Gene Expr Patterns* 2004; **4**: 303–8.
22 Afouda BA, Ciau-Uitz A, Patient R. GATA4, 5 and 6 mediate TGFbeta maintenance of endodermal gene expression in Xenopus embryos. *Development* 2005; **132**: 763–74.
23 Gerber WV, Vokes SA, Zearfoss NR, Krieg PA. A role for the RNA-binding protein, hermes, in the regulation of heart development. *Dev Biol* 2002; **247**: 116–26.
24 Stennard FA, Costa MW, Elliott DA *et al*. Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev Biol* 2003; **262**: 206–24.
25 Small EM, Warkman AS, Wang DZ, Sutherland LB, Olson EN, Krieg PA. Myocardin is sufficient and necessary for cardiac gene expression in Xenopus. *Development* 2005; **132**: 987–97.
26 Brade T, Gessert S, Kühl M, Pandur P. The amphibian second heart field: xenopus islet-1 is required for cardiovascular development. *Dev Biol* 2007; **311**: 297–310.
27 Rones MS, McLaughlin KA, Raffin M, Mercola M. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. *Development* 2000; **127**: 3865–76.
28 Gurdon JB, Mohun TJ, Sharpe CR, Taylor MV. Embryonic induction and muscle gene activation. *Trends Genet* 1989; **5**: 51–6.
29 Small EM, Krieg PA. Transgenic analysis of the atrialnatriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of ANF. *Dev Biol* 2003; **261**: 116–31.
30 Eroshkin FM, Ermakova GV, Bayramov AV, Zaraisky AG. Multiple noggins in vertebrate genome: cloning and expression of noggin2 and noggin4 in *Xenopus laevis*. *Gene Expr Patterns* 2006; **6**: 180–6.
31 Xiong JW. Molecular and developmental biology of the hemangioblast. *Dev Dyn* 2008; **237**: 1218–31.
32 Shalaby F, Rossant J, Yamaguchi TP *et al*. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995; **376**: 62–6.
33 Gering M, Yamada Y, Rabbitts TH, Patient RK. Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. *Development* 2003; **130**: 6187–99.
34 Green JB, Smith JC. Graded changes in dose of a Xenopus activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* 1990; **347**: 391–4.
35 Ariizumi T, Sawamura K, Uchiyama H, Asashima M. Dose and time-dependent mesoderm induction and

outgrowth formation by activin A in *Xenopus laevis*. *Int J Dev Biol* 1991; **35**: 407–14.
36 Nagamine K, Furue M, Fukui A, Asashima M. Induction of cells expressing vascular endothelium markers from undifferentiated *Xenopus* presumptive ectoderm by co-treatment with activin and angiopoietin-2. *Zoolog Sci* 2005; **22**: 755–61.
37 Devic E, Paquereau L, Vernier P, Knibiehler B, Audigier Y. Expression of a new G protein-coupled receptor X-msr is associated with an endothelial lineage in *Xenopus laevis*. *Mech Dev* 1996; **59**: 129–40.
38 Iraha F, Saito Y, Yoshida K *et al*. Common and distinct signals specify the distribution of blood and vascular cell lineages in *Xenopus laevis* embryos. *Dev Growth Differ* 2002; **44**: 395–407.
39 Zon LI, Mather C, Burgess S, Bolce ME, Harland RM, Orkin SH. Expression of GATA-binding proteins during embryonic development in *Xenopus laevis*. *Proc Natl Acad Sci USA* 1991; **88**: 10642–6.
40 Nagamine K, Matsuda A, Asashima M, Hori T. XRASGRP2 expression during early development of Xenopus embryos. *Biochem Biophys Res Commun* 2008; **372**: 886–91.
41 Suzuki K, Takahashi S, Haramoto Y *et al*. XRASGRP2 is essential for blood vessel formation during Xenopus development. *Int J Dev Biol* 2009 (in press).
42 Liu F, Walmsley M, Rodaway A, Patient R. Fli1 acts at the top of the transcriptional network driving blood and endothelial development. *Curr Biol* 2008; **18**: 1234–40.
43 Inui M, Asashima M. A novel gene, Ami is expressed in vascular tissue in *Xenopus laevis*. *Gene Expr Patterns* 2006; **6**: 613–19.
44 Moriya H, Uchiyama H, Asashima M. Induction of pronephric tubules by activin and retinoic acid in presumptive ectoderm of *Xenopus laevis*. *Dev Growth Differ* 1993; **35**: 123–8.
45 Osafune K, Nishinakamura R, Komazaki S, Asashima M. *In vitro* induction of the pronephric duct in Xenopus explants. *Dev Growth Differ* 2002; **44**: 161–7.
46 Brennan HC, Nijjar S, Jones EA. The specification and growth factor induciblity of the pronephric glomus in *Xenopus laevis*. *Development* 1999; **126**: 5847–56.
47 Uochi T, Asashima M. Sequential gene expression during pronephric tubule formation in vitro in Xenopus ectoderm. *Dev Growth Differ* 1996; **38**: 625–35.
48 Chan T, Ariizumi T, Asashima M. A model system for organ engineering: transplantation of *in vitro* induced embryonic kidney. *Naturwiss* 1999; **86**: 224–7.
49 Kim D, Dressler GR. Nephrogenic factors promote differentiation of mouse embryonic stem cells into renal epithelia. *J Am Soc Nephrol* 2005; **16**: 3527–34.
50 Vigneau C, Polgar K, Striker G *et al*. Mouse embryonic stem cell-derived embryoid bodies generate progenitors that integrate long term into renal proximal tubules *in vivo*. *J Am Soc Nephrol* **18**: 1709–20.
51 Onuma Y, Nishinakamura R, Takahashi S, Yokota T, Asashima M. Molecular cloning of a novel *Xenopus* spalt gene (Xsal-3). *Biochem Biophys Res Commun* 1999; **14** (264): 151–6.
52 Nishinakamura R, Matsumoto Y, Nakao K *et al*. Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* 2001; **128**: 3105–15.
53 Nishinakamura R, Osafune K. Essential roles of Sall family genes in kidney development. *J Physiol Sci* 2006; **56**: 131–6.
54 Moriya N, Komazaki S, Takahashi S, Yokota C, Asashima M. *In vitro* pancreas formation from *Xenopus* ectoderm treated with activin and retinoic acid. *Dev Growth Differ* 2000; **42**: 593–602.
55 Moriya N, Komazaki S, Asashima M. *In vitro* organogenesis of pancreas in *Xenopus laevis* dorsal lips treated with retinoic acid. *Dev Growth Differ* 2000; **42**: 175–85.
56 Chan TC, Takahashi S, Asashima M. A role for Xlim-1 in pronephros development in *Xenopus laevis*. *Dev Biol* 2000; **228**: 256–69.
57 Shi Y, Hou L, Tang F *et al*. Inducing embryonic stem cells to differentiate into pancreatic beta cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* 2005; **23**: 656–62.
58 Jiang J, Au M, Lu K *et al*. Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* 2007; **25**: 1940–53.
59 Jiang W, Shi Y, Zhao D *et al*. *In vitro* derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Res* 2007; **17**: 333–44.
60 Nakanishi M, Hamazaki TS, Komazaki S, Okochi H, Asashima M. Pancreatic tissue formation from murine embryonic stem cells *in vitro*. *Differentiation* 2007; **75**: 1–11.
61 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154–6.
62 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981; **78**: 7634–8.
63 Nakanishi M, Kurisaki A, Hayashi Y *et al*. Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. *FASEB J* 2009; **23**: 114–22.
64 Honda M, Kurisaki A, Ohnuma K, Okochi H, Hamazaki TS, Asashima M. N-cadherin is a useful marker for the progenitor of cardiomyocytes differentiated from mouse ES cells in serum-free condition. *Biochem Biophys Res Commun* 2006; **351**: 877–82.
65 Nishimura Y, Hamazaki TS, Komazaki S, Kamimura S, Okochi H, Asashima M. Ciliated cells differentiated from mouse embryonic stem cells. *Stem Cells* 2006; **24**: 1381–8.

66 Weaver M, Yingling JM, Dunn NR, Bellusci S, Hogan BL. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* 1999; **126**: 4005–15.
67 Takahashi K, Tanabe K, Ohnuki M *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861–72.
68 Yu J, Vodyanik MA, Smuga-Otto K *et al*. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917–20.
69 Intoh A, Kurisaki A, Fukuda H, Asashima M. Separation with zwitterionic hydrophilic interaction liquid chromatography improves protein identification by matrix-assisted laser desorption/ionization-based proteomic analysis. *Biomed Chromatogr* 2009; **23**: 607–14.
70 Intoh A, Kurisaki A, Yamanaka Y *et al*. Proteomic analysis of membrane proteins expressed specifically in pluripotent murine embryonic stem cells. *Proteomics* 2009; **9**: 126–37.