Investigating the mechanisms of cardiac patterning and morphogenesis using a heart formation assay

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Abstract

Vertebrate heart development involves a precise sequence of morphogenetic events from which a complex structure is formed from a linear heart tube. To study the heart development in mammals is difficult because most alterations of heart structure are lethal. Therefore we use alternative model, *Xenopus laevis* embryos. The aim of this project is try to establish a new experimental model to help understanding the mechanism that regulates cardiac cell diversification and heart morphogenesis. In order to achieve these goals we use two assays. The cardiogenesis assay involves the use of animal cap explants excised from the animal pole of blastula embryos. It has been previously established that it is possible to induce differentiation of cardiac tissue in the same explants via the injection of GATA-4 mRNA. Here it is shown that GATA-4 reliably induces the expression of ventricular and proepicardial markers, providing an assay to study the mechanisms of cardiac cell fate diversification. However, despite these, cardiomyocytes generated in animal pole explants they do not undergo significant morphogenesis and physiological maturation. In order to study these later aspects of heart development we required a different assay in which was possible to generate a structure similar to the heart. Using GATA-4 injected AC explants transplanted into host embryos we obtained secondary beating hearts in which regionally restricted cardiac gene expression was observed in addition to growth and a limited degree of morphogenesis. We demonstrated that the host plays an essential role as it provides a wide range of permissive regions which allow the development of the SH. Moreover, we also showed that the competence to generate a secondary heart is lost in reaggregates transplanted at stage 28. The host cells however do not contribute to the SH indicating that the role of the host is providing signals which allow the development of the SH. In the future we aim to investigate the signalling pathways which mediate the host-SH interaction and the mechanism by which they allow the development of the secondary structure.

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List of abbreviation

A	atria
AC	Animal Caps
Alk	Activin receptor like kinase
ANF	Atria Natriuretic Factor
A-P	Anterior-Posterior
BB/BA	Benzyl alcohol; Benzyl benzoate
BCNE	Blastula Chordin and Noggin expression centre
BMP	Bone Morphogenic Protein
C	Control
cTnI	Cardiac Troponin
CMFM	Calcium Magnesium-Free Media
Dex	Dexamethasone
dH_2O	Distilled Water
ddH_2O	Double Distilled Water
Dkk-1	Dickopf-1
DMSO	Dimethyl Sulphoxide
dNTP	deoxyribonucleotide triphosphate
D-V	Dorso-Ventral
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Fig	Figure
G4	GATA-4

GR	Glucocorticoid Receptor
H	Host
HF	Heart Field
HFA	Heart Field Assay
IH	interhyoid muscles of the jaw
IMHC	Immuno hystochemestry
Irx4	Iroquois
L-R	Left-Right
LH	limph heart
LPM	Lateral Plate Mesoderm
MAB-T	Maleic Acid Buffer-Triton
MBT	Mid Blastula Transition
Mef	Myocyte enhancer factor
MEMFA	MOPS-EDTA-Magnesium Formaldahyde
min	minutes
ml	millilitre
MLC2	Myosin Light Chain 2
MLC1v	Ventricular Myosin Light Chain 1
NAM	Normal Amphibian Media
OFT	Outflow tract
ODC	Ornithine Decarboxylase
pg	picogram
QRT-PCR	Quantitative Reverse Transcriptase PCR
R	Reaggregate

RA	Retinoic Acid
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SH	Secondary Heart
St.	Stage of development
tBr	Truncated BMP receptor
Tbx	T-box
TBS	Tris Buffer Saline
TTw	Tris Buffered Saline
TGFβ	Transforming Growth Factor
UV	Ultra Violet
V	ventricle
WE	Whole Embryos
WMISH	Whole Mount in situ Hybridization
Wnt	Wingless-type MMTV integration site
Xnr	*Xenopus* nodal related
µg	microgram
µl	microlitre
µM	micromolar

1. *Introduction*

1.0 Introduction

Embryonic development is regulated by numerous inductive signals that determine specification of different cell types. Specification is a process through which naive cells are induced to enter a developmental pathway. A specified cell may not ultimately form the tissue that it has been directed toward until it is determined, by which point the specified cell expresses cell-specific proteins that confirm its particular identity (Slack, 1991). Following specification, functionally distinct differentiated cells interact to generate a specific organ during morphogenesis.

1.1 Embryonic development of Xenopus laevis

1.1.1 Xenopus laevis embryo as a model organism

The African clawed toad *Xenopus laevis* is one of the major animal models used in developmental biology. In the first half of 20^{th} century it became popular after it has been discovered that ovulation in female *Xenopus* can be induced by the injection of the human hormone chorionic gonadotropin (HcG). This permitted a constant supply of embryos in contrast to the seasonal availability of other amphibian species as urodele and newt.

Advantages in using frogs in research include large and robust embryos that are available in large numbers. Development takes place outside the mother and as such embryos are accessible at all well-documented developmental stages (fig. 1.1) for experimental procedures. Examples of contribution of *Xenopus* as a model for developmental study are numerous and

include the generation of embryonic fate, specification maps and discovery of the mechanisms that specify embryonic axes (Beck and Slack, 2001). Gene function analysis can be easily achieved by overexpression of mRNA in early cleavage embryos. The capacity of *Xenopus* embryo to translate the mRNA injected into the blastomeres has led to many contributions to the study of the early developmental events and the identification of the major classes of inducing factors. Gene function analysis can be also achieved through gene knockdown using antisense morpholino (MO). MOs are designed to block the mRNA translation or to block specific region of the gene avoiding the splicing event of the pre-mRNA (Heasman, 2002). *Xenopus* is also used to study the late events in embryo development using the transgenic technology. Additional benefit of transgenesis is the ability to study the expression of a target gene using integrated transgenes as green fluorescent protein (GFP) in living embryos. GFP is used to study the regulation of gene expression and as a real time marker of cell fate. More recently the use of an model organism database, Xenbase, has become the principal resource for gene expression analysis, anatomy, development and community information for *Xenopus laevis* and *Xenopus tropicalis* (Bowes *et al.*, 2010).

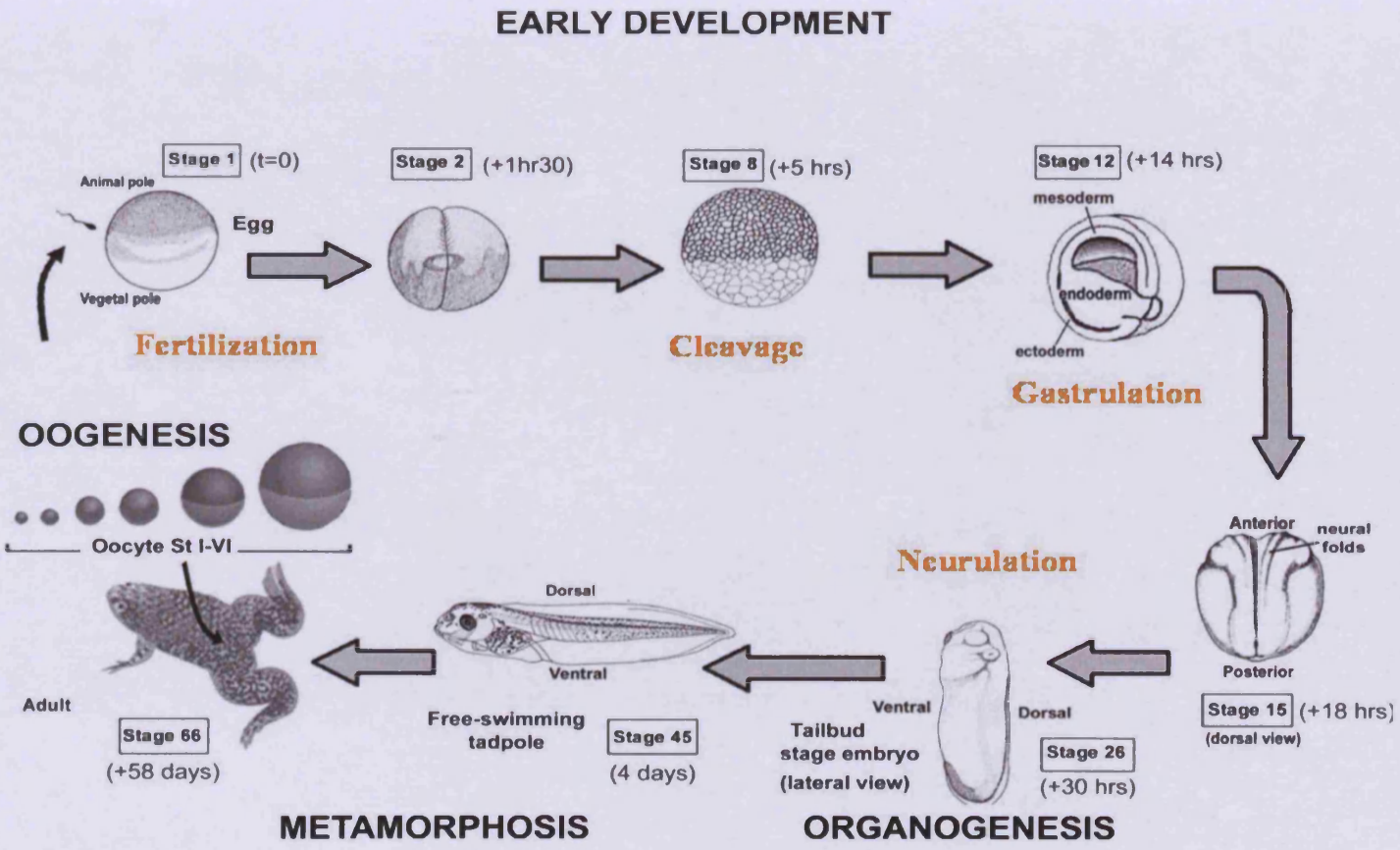

Figure 1.1 Life cycle of Xenopus laevis. Modified from (Mereau *et al.*, 2007), *Xenopus* life cycle is composed by different and critical developmental steps starting from fertilization followed by cleavage and gastrulation in which three embryonic tissues are developed. Neurula tube formation is followed by organogenesis which ends after four day with the metamorphosis. Adult frog becomes sexually mature after 12 months.

1.1.2 Early events in Xenopus development

The unfertilized egg has heavily pigmented animal pole and unpigmented vegetal pole. The animal-vegetal division represents the first axis in the egg before fertilization. Fertilization can occur anywhere in the animal pole, but the point of the sperm entry is important to generate the first embryonic axis, the dorsal-ventral (D-V) axis. The first step after fertilization is characterised by a series of cell divisions between the animal and the vegetal pole in a phase called Cleavage. Upon the 12^{th} cell cycle (4000-cells stage) the zygotic gene transcription starts with the inclusion of the G-phase in mitosis due to the demethylation of the genomic DNA. This phase is called Mid Blastula Transition (MBT). At the 15^{th} cycle the frequency of cleavage slows down due to the stop of the mitosis and shortly afterwards the embryo begins to gastrulate (Heasman, 2006). During gastrulation the morphology of the embryo is reorganised to form the three germ layers: the ectoderm, precursor of epidermis and nervous system, the endoderm, precursor of the epithelium of the digestive tube and its associated organs and mesoderm, precursor of blood, heart, muscle, kidney, gonads, bone and connective tissue (Gilbert, 2006; Heasman, 2006). Each germ layer gives rise to different cell types which are not randomly distributed in the embryo but organised into complex tissues and organs. The pattern distribution of different organs is a process regulated via inductive cell-cell interaction and it depends directly from the embryo patterning and the establishment of the body axis.

1.1.3 Embryonic axis formation

During the early development two main body axes are established, the dorsoventral axis (D-V) the anteroposterior axis (A-V) (De Robertis and Kuroda, 2004). Dorsoventral axis is established in the embryos by migration of the dorsal determinants (Weaver and Kimelman, 2004). Dorsal determinants are composed of the components of Wingless-type MMTV integration site (Wnt) signalling (reviewed in section 1.1.5.3). During oogenesis Wnt pathway components GSK3-binding protein GBP and Dishevelled (Dsh) lie in the vegetal pole of the embryo (Weaver and Kimelman, 2004). After sperm enters the animal pole, embryogenesis is activated and egg cortex rotates 30° with respect to the internal cytoplasm (fig. 1.2 B). The rotation is driven by microtubules nucleated by the centriole. The microtubule movement mediated via kinesis dependent motor serves as a truck on which molecules as Dsh, GBP can move dorsally. Once located in the dorsal side of the embryo GBP and Dsh regulate the activation of β-catenin signal which accumulates dorsally and becomes localised in the nuclei with the peak level during mid-blastula stages (fig. 1.2 A) (De Robertis and Kuroda, 2004; Rowning *et al.*, 1997; Schneider *et al.*, 1996; Weaver and Kimelman, 2004).

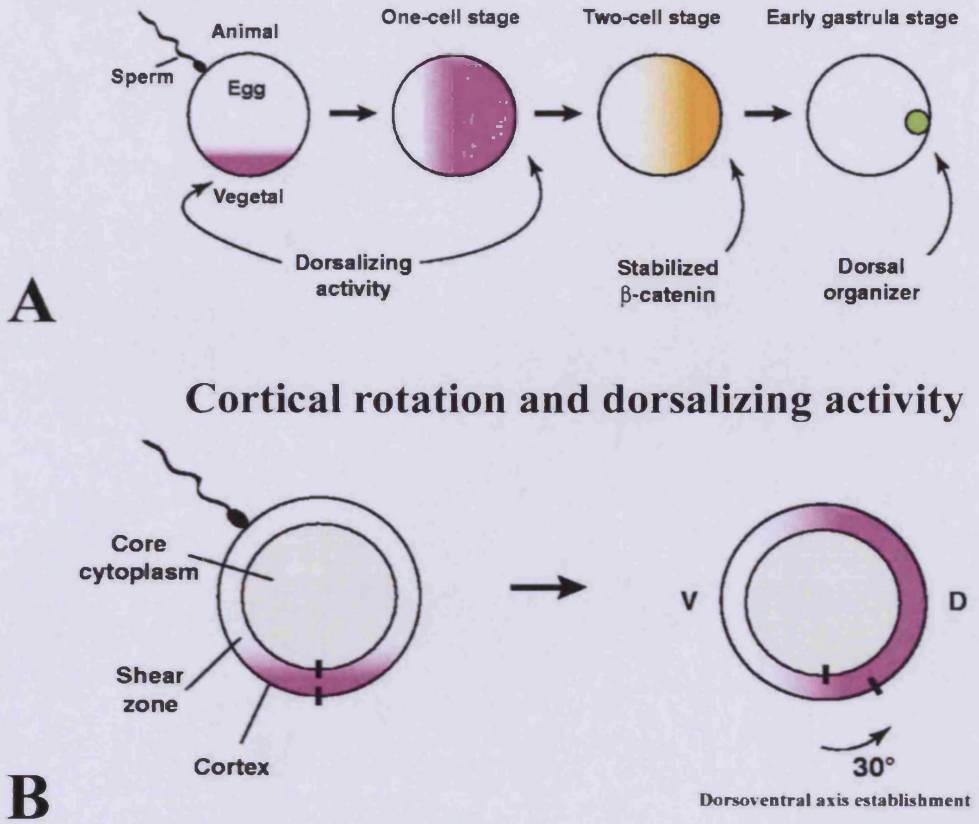

Figure 1.2 *Xenopus* dorsoventral axis formation. Modified from Weaver and Kimelman, 2004. (A) Sperm entry in the animal pole generates a translocation of the dorsalising activity which leads to β-catenin stabilization in the dorsal side in the opposite point of the sperm entry. By the two cell-stage the maternal β-catenin has become asymmetrically stabilised in the dorsal region where activate genes of the dorsal organiser or Spemann's Organiser. (B) The cortical rotation moves in the same direction as the dorsal determinants which initially are located in the shear zone between the outer cortex and the core cytoplasm. The black bars at the vegetal pole show the position of the core and the outer cortex before the fertilisation occurs. After the sperm entry, the outer cortex moves 30° with respect to the core toward the dorsal side together with the dorsal determinants.

After fertilization, Wnt-11 mRNA is vegetally localised and during the cortical rotation movements becomes enriched in the dorsal side of the embryo (Tao *et al.*, 2005). During cleavage stages, Wnt-11 activates signalling via the canonical Wnt-pathway. β-catenin is therefore synthesised and interacts with Xtcf3 in dorsal nuclei to activate Wnt target genes after MBT (Tao *et al.*, 2005). The activation of the dorsal signalling pathway is also regulated from Wnt-5a signalling which together with Wnt-11 activate the canonical β-catenin and non-canonical JNK pathways in early embryo. Maternal Dkk-1 antagonises both the canonical and non-canonical Wnt pathways to regulate the D-V axis formation (Cha *et al.*, 2008). The importance of the microtubule transport of the dorsal determinant has been demonstrated using *Xenopus* embryo exposed to the UV light. Embryo exposed to the UV irradiation in the vegetal pole lacks of the dorsal structures resulting completely ventralised. The ventralised phenotype is obtained due to the disruption in the microtubule assembly which prevents the migration of dorsal determinants (Weaver and Kimelman, 2004). Therefore, β-catenin localisation in the dorsal side is abolished and causes the accumulation of β-catenin in the nuclei of the vegetal pole (Schneider *et al.*, 1996). Ventralised embryo phenotype can be easily rescued by injection of synthetic mRNAs encoding, for instance, chordin (BMP antagonist, section 1.1.5.1) or mediators of Wnt and Nodal pathways. Injections of Wnts, Nodal or BMP antagonist generate a region of low BMP signalling that is normally required for formation the dorsal structure.(De Robertis *et al.*, 2000).At mid-blastula stages β-catenin nuclei localization in the dorsal side of the embryo determines the generation of two signalling centres, the Blastula Chordin and Noggin Expression centre (BCNE centre) centre in the animal pole and the Nieuwkoop centre in the vegetal region (fig. 1.3) (De Robertis and Kuroda, 2004). BCNE_(De Robertis and Kuroda, 2004) is involved in the formation of the anterior neural tissue and expresses Nodal-related protein Xnr-3 and BMP antagonist *Chordin*

and *Noggin*. At gastrula stage, the same set of genes is expressed in the signalling centre Spemann's Organiser under the control of Nodal-related signals (fig. 1.3). The Nieuwkoop centre is established in the vegetal pole through the action of β-catenin gradient and the maternal factors VegT (T-box transcription factor) and Vg1 (Transforming growth factor β) expression. VegT induces the expression of potent mesoderm inducers *Xenopus* Nodal-related factors (Xnr1, 2, 4, 5 and 6) (fig. 1.3). The levels of Xnrs are further increased in the area of high Wnt signalling, in the Nieuwkoop Centre, thereby creating a gradient. During late blastula stages high levels of Nodal on the dorsal side of the embryo lead to the establishment of the Spemann's Organiser. Low levels of Nodal lead the formation of the ventral mesoderm (De Robertis and Kuroda, 2004; De Robertis *et al.*, 2000). Maternal control of the pattern formation occurs before the establishment of the three germ layers and is fundamental in the embryonic pre-pattern formation before the zygotic gene transcription occurs. The key proteins involved are VegT, Vg1 and Wnt-11. The role of these factors has been established using depletion of maternal and zygotic protein models during embryo development (Heasman, 2006; White and Heasman, 2008). VegT depleted embryos do not form endoderm, show reduced mesoderm-inducing signal and lack of gastrulation movements. Similarly, Vg1 is required for proper endoderm development and mesoderm induction (White and Heasman, 2008). Wnt-11 depleted embryos fail to form the neural folds and are ventralised demonstrating the important role of Wnt-11 in the dorsal axis formation. Maternal control appears to be essential for the establishment of pre-patterning and is the key regulator of the zygotic genes activation at the MBT (Heasman, 2006).

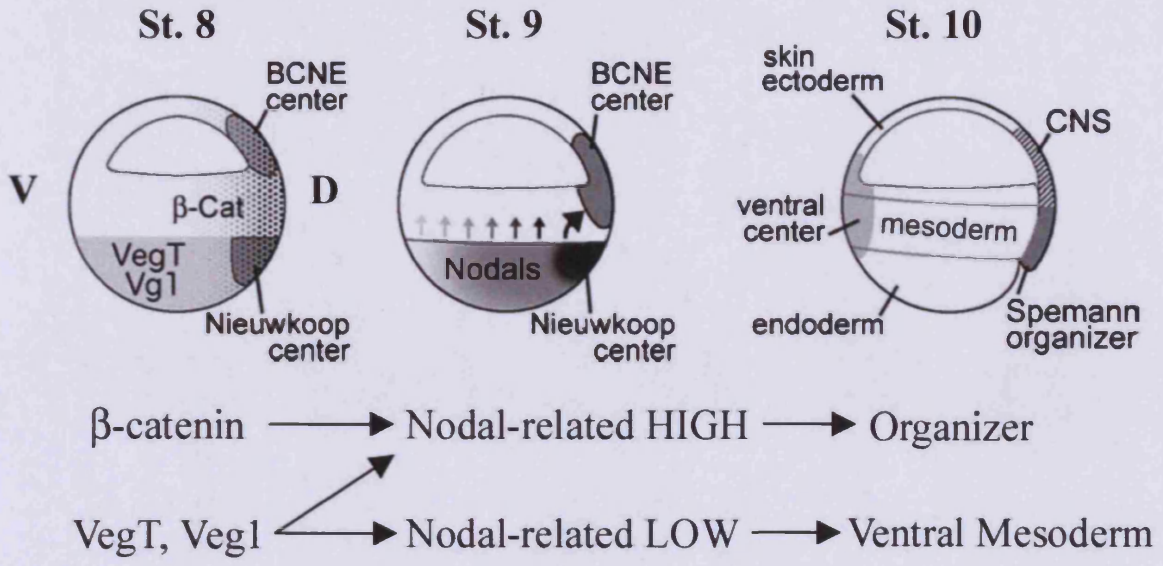

Figure 1.3 Organizer and three germ layers formation. Modified from De Robertis *et al.*, 2004 and Agius *et at.*, 2000. At blastula stage (st.8) the embryo shows the Nieuwkoop centre in the vegetal pole and the BCNE centre in the animal pole. Both are dependent from the β-catenin localization in the dorsal side. The Nieuwkoop centre is located in the junction between β-catenin and VegT and Vg1 gene expression and BCNE is involved in the formation of the anterior neural tissue via the expression of *Chordin*, *Noggin* and *Xnr-3*. Higher β-catenin signal in the dorsal side and the expression of VegT and Vg1 in the ventral side generates a high gradient of nodal signal in the endoderm where the Nieuwkoop centre is located (st.9). This gradient induces the formation of the overlying mesoderm. During gastrula stage (st.10) high level of nodal generates the Spemann's organiser in the dorsal side while low level of nodal lead the formation of the ventral mesoderm. Thus, Nieuwkoop centre is the region of the dorsal endoderm that induces the organiser tissue (Agius *et al.*, 2000; De Robertis *et al.*, 2000).

1.1.4 Mesoderm Specification

Mesoderm induction was identified by Nieuwkoop and colleagues using late blastula embryo animal cap explants (AC) juxtaposed with explants of the vegetal pole (Nieuwkoop, 1969). AC cells are not under the influence of mesoderm-inducing signal and if cultured alone generate atypical epidermis. In the presence of the vegetal pole, mesoderm induction occurs with the development of a variety of mesoderm cell types such as muscle, notochord and blood (Nieuwkoop, 1969). During the blastula stage, vegetal endoderm secretes mesoderm-inducing signals conferring dorso-ventral pattern to the mesoderm (fig. 1.4). Dale and Slack, 1987 demonstrated that the ventral and lateral vegetal pole induces the ventral mesoderm (mesenchyme, blood, and small amount of muscle) and dorsal vegetal cells induce dorsal mesoderm cell types such as notochord and muscle (Dale and Slack, 1987). An additional source of signal (the third signal in 3 signal model) originates from the Spemann's Organiser centre during gastrulation (fig. 1.4). When the dorsal mesoderm has been induced, it dorsalises the ventral mesoderm to form the intermediate mesoderm which gives rise to the muscle progenitor cells (Dale and Slack, 1987; Kessler and Melton, 1994; Smith, 1989; Smith, 1993). The cells in the ventral midline outside the dorsalising activity generate only blood tissue (Smith, 1989). During gastrulation, Spemann's Organiser acts as a signalling centre via the release of antagonists of growth factors to the adjacent cells in order to organise the embryonic axis (De Robertis *et al.*, 2000). For example, the activity of the BMP antagonists *Noggin* and *Chordin* is essential for the ventral mesoderm formation where BMP signal is highly expressed (Graff, 1997). The third set of signals therefore, antagonises the ventralising action of the fourth set of signals (updated as 4 signal model) which comes from the ventral region of the embryo (fig. 1.4) (Wolpert *et al.*, 2007).

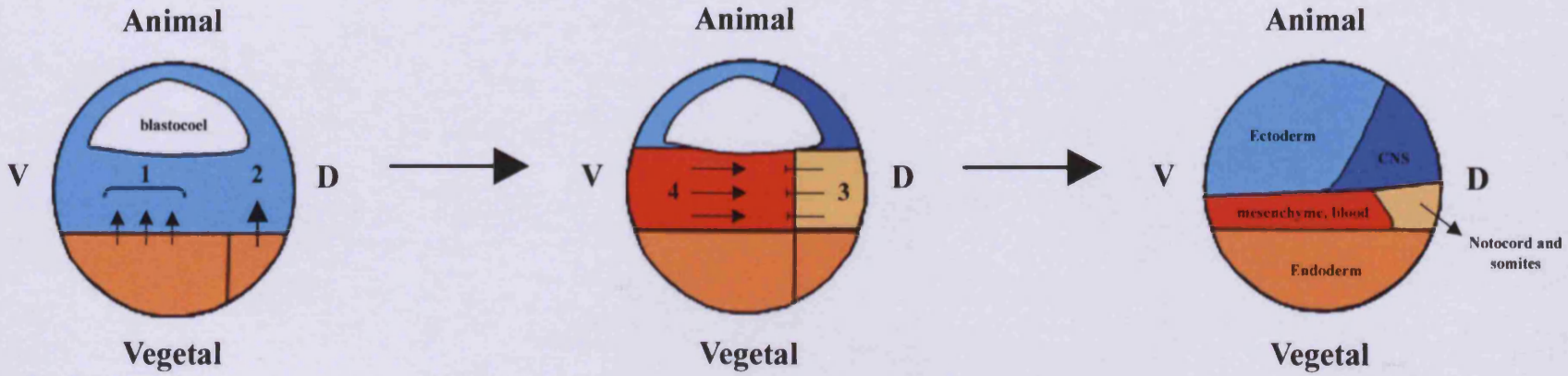

Figure 1.4 The four signal model in mesoderm induction. Adapted from Wolpert, 2007. Mesoderm induction in *Xenopus* involves four set of signals. The first two signals originate in the vegetal region of the embryo. The first set of signals (1) comes from the ventral region to specify a ventral-type mesoderm. The second set of signals (2) comes from the Nieuwkoop centre and specifies the dorsal-most mesoderm that will form the Spemann's organiser. The third set of signals (3) emanates from the Spemann's organiser and inhibits the ventralising action of the forth set of signals (4) which comes from the ventral region (Wolpert *et al.*, 2007).

The fourth set of signal is secreted to ventralise the mesoderm and interact with the dorsalising signal. Thus, the establishment of a pattering in the mesoderm along the D-V axis depends on a balance between the dorsaling and the ventralising signals and the capacity of the cells to respond differently in relation of their position along the morphogen gradient.

1.1.5 Mesoderm inducing factors

Embryonic induction is regulated by a set of signalling pathways which are evolutionary conserved. These signals regulate cell-cell contact, tissue specification and organogenesis. In particular, the pathways described below are involved in mesoderm induction, axis specification and embryo pattering.

1.1.5.1 TGF-β family members

TGF-β family is composed of a large group of proteins including activin/inhibin family, Bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs) and the TGF-beta subfamily. Those members play fundamental roles in the regulation of various biological processes such as cell proliferation, lineage determination, differentiation, apoptosis, cell migration and adhesion (Massague, 1998). Members of the TGF-beta superfamily interact with a conserved family of transmembrane protein serine/threonine-specific kinases referred to as the TGF-β receptor family (Massague, 1998). Ligands bind the receptor type II which recruits and phosphorylate the receptor type I. Receptor type I phosporylates and activates the intracellular Regulatory Smad (RSmad; Smad-1, -2, -3, -5 and -7). RSmad accumulates in the

nucleus as heteromeric complex with a second class of Smads, the common Smad (coSmad4). The complex RSmad/coSmad-4 associates with DNA binding proteins and various transcriptional coactivators or corepressors to regulate positively or negatively gene expression (Attisano and Wrana, 2002). Vertebrates have seven distinct type I receptors, each of which can bind with one of five type II receptor to mediate signals for the TGF-β family lingand (Attisano and Wrana, 2002). Activin/nodal signalling is activated via the interaction with the Activin receptor-Like Kinase (ALK) 4 and 7 with the following phosphorylation of Smad-2 and -3. BMP bind BMPR-IA and IB receptor with the following phosphorylation of Smad-1, -5 and -8. Both pathways transduce the signal via coSmad-4 (Attisano and Wrana, 2002; Massague, 1998).

In *Xenopus,* after MBT different TGFβ members are involved in the process of embryonic induction. Those members involve a subfamily functionally related to activin which includes *activin*, *derriere*, and the *Xenopus* nodal related genes Xnr-1, -2, -4, -5 and -6 and members of the BMP subfamily BMP-2, -4 and -7 (Hill, 2001). Activin/nodal family act in mesoderm induction (Wardle and Smith, 2006), endoderm formation and gastrulation movements (Hill, 2001). The involvement of activin as a mesoderm inducer has been shown in animal cap explants assay. Explants treated with activin generate a variety of dorsal and ventral mesoderm tissues (Ariizumi and Asashima, 2001; Ariizumi *et al.*, 1991; Asashima *et al.*, 1990; Green *et al.*, 1990). The mesodermal inducing activity is dependent from the concentration used in AC explants. Low activin concentration induces the formation of ventral type mesoderm such as blood-like cells and mesenchyme, while high concentration induce the formation of dorsal-type mesoderm such as muscle and notochord (Ariizumi *et al.*, 1991; Green *et al.*, 1992). The cells respond to the factor according to predetermined threshold of concentration. Therefore, activin can be defined as a morphogen due the ability to induce different cell types. In

embryos, activin subfamily members are believed to be produced from the vegetal cells and their synthesis require the maternal transcription factor VegT (Kofron *et al.*, 1999). The *Xenopus* Nodal-related proteins Xnr-5 and -6 are the first mesoendodermal inducing factors expressed before the MBT under the regulation of the β-catenin Tcf complex (Yang *et al.*, 2002). Xnr-1, -2 and -4 are expressed slightly later in the dorsal side of the embryos (Takahashi *et al.*, 2000). Agius and colleagues, suggested a model (fig. 1.3) for the mesoderm induction where high level of Nodal in the dorsal side of the embryo induces the formation of the Spemann's organiser, while low level of Nodal in the ventral side induces the formation of the ventral mesoderm (fig. 1.3) (Agius *et al.*, 2000).

Bone morphogenic proteins (BMP) exhibit a gradient of distribution in the mesoderm. In the prospective ventral mesoderm and ectoderm there are high levels of BMP while in the dorsal side there are low levels (Graff, 1997). It has been shown that BMP-4 is a stronger inducer of the ventral mesoderm together with BMP-2 and -7 (Graff, 1997; Hill, 2001). The high level of BMP activity on the ventral side is modulated by two potent inhibitors *Chordin* and *Noggin* which are expressed in the dorsal marginal zone during gastrulation. *Chordin* and *Noggin* bind with high affinity BMP-4 and -2 abolishing BMP activity by inhibiting the binding of the ligand to the receptor (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Thus, the dorsal fate is promoted from the inhibition of the ventral molecules from the organiser antagonist rather the active induction of the dorsal fate.

1.1.5.2 Fibroblast growth factor family

Fibroblast growth factors (FGF) constitute a large family composed of 22 members in mammals. FGFs are involved in a wide range of cellular process including early embryonic development, chemotaxis, cell migration, differentiation and apoptosis (Bottcher and Niehrs, 2005). FGF proteins bind the receptor (FGFRs) which belong to a subfamily of the cell surface receptor tyrosin kinases. FGF binding causes a dimerisation of the receptor and the autophosphorylation of the intracellular domain which is fundamental to recruit the signal complex. Signal transduction occurs via Ras/MAPK pathway, but also via the PI_3-kinase/Akt or PLCγ pathway (Bottcher and Niehrs, 2005). FGF plays an important role as mesoderm inducer. FGF was the first purified molecule demonstrated to induce mesoderm in animal cap explants (Slack *et al.*, 1987). The high expression of FGF in the lateral and ventral vegetal region of *Xenopus* embryos generates a gradient in the mesoderm along the D-V axis (Smith, 1993). Thus, FGF proteins induce the ventrolateral mesoderm to form the mesenchyme and small amount of muscle. Green and colleagues, proposed a model of orthogonal concentration gradients of activin and FGF throughout the marginal zone. This generates a gradient of signal, in which cellular responses are bound by tight thresholds of activin concentrations that are modified by FGF which in turn patterns the mesoderm during embryonic development (Green *et al.*, 1992). FGF mesodermal target is the T-box transcription factor *brachyury* (*Xbra*) which is required for the posterior mesoderm and axial development in the mouse, zebrafish and *Xenopus*. In *Xenopus* *Xbra* is expressed transiently in the presumptive mesoderm and persistently in the notochord and in the posterior tissues. Mesoderm induction requires FGF pathway downstream target gene *brachyury*. Both are components of a regulatory loop in which *Xbra* induce FGF and vice versa. This regulatory loop is required for

mesoderm inducing activity of *Xbra*. If FGF activity is disrupted, by interfering with FGF signalling, mesoderm formation is blocked (Schulte-Merker and Smith, 1995).

1.1.5.3 Wnt signalling pathway

Wnts are a large family of proteins involved in different cellular mechanisms such as embryonic development, tumour progression and stem cell differentiation (Eisenberg and Eisenberg, 2007). The canonical Wnt pathway transduces the signal via the binding of the frizzled family of transmembrane receptor and the lipoprotein receptor-related (LRP) isoform LRP5 and LRP6. The interaction ligand/receptor, relayed via the cytoplasmatic protein Dishevelled (Dsh), promotes the inactivation of glycogen synthase kinase 3 (GSK3) with a consequent accumulation of β-catenin. β-catenin translocate into the nucleus and forms a transcriptional enhancer complex with LEF/TCF DNA binding protein. Wnt signalling plays an important role during embryogenesis. In particular, components of the Wnt pathway such as β-catenin, GSK and Dsh are enriched dorsally during the first cell cycle to establish embryonic D-V axis (reviewed in section 1.1.3). During the early germ layer formation, Wnt signal is required for the formation of the ventral mesoderm. Wnt8 is expressed in the ventral and lateral mesoderm after MBT but not in the cells of the Spemann's organiser. The repression of Wnt-8 expression in the organiser is important for normal dorsal development (Christian and Moon, 1993). Ectopic expression of Wnt-8 after mid-blastula stage promotes the differentiation of isolated AC as ventral mesoderm. Thus, Wnt-8 is involved in the differentiation of the ventral mesoderm after the MBT (Christian and Moon, 1993). However, it has been shown that overexpression of Wnt-8 at cleavage stage embryo does not cause autonomous mesodermal differentiation in isolated AC explants (Christian *et al.*, 1992).

Primary induction of the mesoderm has a maternal origin and start before the onset of the zygotic gene expression. Therefore, Wnt-8 does not act as a primary mesoderm inducing signal because it is expressed after MBT to direct marginal zone cells to differentiate as ventral mesoderm (Christian and Moon, 1993). During gastrula stage dorsoventral pattering require two distinct signals. The dorsalising signal from the Spemann's organiser where Wnt antagonist Dkk-1 (Glinka *et al.*, 1998) and FrzB (Leyns *et al.*, 1997) create a zone of low levels of Wnt signal to induce the embryo dorsalisation and the development of the head. The second signal initiates the ventral mesoderm differentiation via Wnt-8 expression and attenuates the response of the cell to the Organiser inducing signals. Therefore, the dorsoventral patterning in the embryonic mesoderm is determined by a balance of Wnt signal inhibitors from the dorsal side and Wnt expression in the ventral side (Christian and Moon, 1993).

1.2 Heart development

During development mesoderm differentiates into chordamesoderm, paraxial mesoderm, intermediate mesoderm and lateral mesoderm.

The notochord originates from chordamesoderm, also known as axial mesoderm, which is replaced by the vertebral column. The paraxial mesoderm, located along both sides of the neural tube, is the precursor of the somites. The intermediate mesoderm, located between the paraxial mesoderm and the lateral plate, is the precursor of the urogenital system, kidneys and gonads, and lateral plate mesoderm, located in the periphery of the embryo, becomes divided horizontally into two layers; the somatic layer (under the ectoderm) that will form the future body wall and the splanchnic mesoderm (above the endoderm) that will form the future circulatory system, viscera and heart. The space between these two layers is called the coelom. The early developing heart is therefore of mesodermal origin. However, late in development of amniotes evidence suggests that there are also contributions from cells of neural crest origin (Gilbert, 2006).

1.2.1 Early events in heart development

Heart development in mouse, chicken, amphibian and zebrafish arises from cells in the anterior lateral plate mesoderm of early embryo which locate in either side of the precordal plate and rostral notochord during gastrulation movements. The specification of the heart precursors occurs via multiple tissue and cell-cell contact which are temporally and spatially regulated by a network of inductive signals. In mammals and birds the bilateral fields of cardiogenic mesoderm fuse in the anterior region of the embryos to generate the cardiac

crescent (Zaffran and Frasch, 2002). In *Xenopus*, the regions which lead the inducing signal to specify cardiac precursor cells have been identified as the dorsal midline mesoderm (Sater and Jacobson, 1990b) also known as Spemann's Organizer (Foley *et al.*, 2006) and anterior endoderm (Nascone and Mercola, 1995). Signals responsible for cardiac specification involves Wnt antagonism, Bone Morphogenic Protein (BMP), Fibroblast Growth Factor (FGF) and TGFβ family member Nodal (Foley *et al.*, 2006; Mohun *et al.*, 2003). These signals interact to specify the cardiogenic cell lineage via the expression of a network of cardiogenic transcription factors (GATA, Nkx, T-box). Those factors are responsible for the transcription of cardiac differentiation markers during the heart morphogenesis. Evidence in chick shows that cardiac inducing activity of endoderm occurs via BMP expression (BMP-2, -4 and -7) (Schultheiss *et al.*, 1997) which overlap with the expression of the vertebrate homolog of the Drosophila *tinman* gene, Nkx2.5 (Schultheiss *et al.*, 1997). In fact, it is believed that BMP and Nkx2.5 interact synergistically to induce myocardial differentiation (Jamali *et al.*, 2001a). BMP activity is necessary but not sufficient to induce cardiogenesis. It has been shown that cardiac induction requires additional factors from the anterior endoderm. These factors include Wnt-β catenin inhibitors Dkk-1 and Crescent which establish a zone of low Wnt activity where cardiogenesis occurs. Thus, it has been proposed that heart formation occurs in the anterior lateral mesoderm by the establishment of a region expressing low levels of Wnt signal and high level of BMP signal (Marvin *et al.*, 2001; Schneider and Mercola, 2001). However, recent evidence shows that the Wnt pathway has no significant effect during cardiac specification but its inhibition is required in a period between the end of specification and the onset of differentiation (Samuel and Latinkic, 2009). Contrary to the other Wnt family members it has been shown that Wnt-11 regulates cardiogenesis. Loss- or gain-of function experiments in *Xenopus* have shown that Wnt-11 is required for heart formation and it is

sufficient to induce an ectopic beating heart in embryonic explants (Pandur *et al.*, 2002). An additional signal required for heart development in zebrafish and chick is FGF8. FGF8 is present, in zebrafish, in the precardiac mesoderm and is required for the ventricular morphogenesis (Marques *et al.*, 2008; Reifers *et al.*, 2000). FGF8 mutant acerebellar (*ace*) lack GATA-4 and Nkx2.5 expression and is defective in the ventricular chamber (Reifers *et al.*, 2000). In chick FGF8 cooperates with BMP2 to induce cardiogenesis. BMP2 upregulates ectopical FGF8 and therefore FGF8 acts downstream of BMP2 (Alsan and Schultheiss, 2002). Evidence in *Xenopus* shows that FGF and Nodal signalling are required during the first hour of cardiac induction at gastrulation (Samuel and Latinkic, 2009).

1.2.2 Heart morphogenesis

Mapping experiments of cardiac progenitor cells in the developing avian and mouse embryo shows that specification of the heart takes place in the primitive streak (posterior to the Hensen's node) from which emerges the cranio-lateral mesoderm at gastrula stage (Buckingham *et al.*, 2005; Harvey, 2002). The cardiac progenitors originate from the primitive streak and migrate to the anterior of the embryo to lie under the head fold forming two groups of cells on either side of the midline (mouse E6.5) (Buckingham *et al.*, 2005). The cells migrate to the ventral midline to form a crescent shaped epithelium also known as the cardiac crescent (mouse E7.5-8) which is the precursor of the atrial and ventricular musculature, the valves and Purkinje fibres. The early heart tube is successively formed from cardiac crescent (Buckingham *et al.*, 2005) and is composed of and inner endothelial tube covered by a myocardial layer (Harvey, 2002). Different analysis showed that the cardiac crescent cells are

pre-patterned, in terms of their contribution to the cardiac tube, along the anterior-posterior axis (Buckingham *et al.*, 2005). Thus, it was believed that the straight heart tube contains all cardiac compartments as circumferential segments (fig. 1.5 A). This theory however, has been recently revisited. It is now firmly established that the heart tube cells are fated to become the left ventricle only and the remaining myocardium is added during the subsequent development (fig. 1.5 A) (van den Berg and Moorman, 2009). The future ventricle cells are positioned more anteriorly in the heart tube with respect to their future location (fig. 1.5 A)(Buckingham *et al.*, 2005). When the elongated heart tube has formed assumes a rightward spiral form, in a process known as heart looping (Fig. 1.5 B). The process of looping converts the original anterior-posterior patterning of the heart tube into the left-right asymmetry which is finally resolved with the chamber formation (fig. 1.5 B) (Fishman and Olson, 1997). The complexity of understanding the chamber formation has become more difficult since it has been discovered that not all cardiac chambers are present in the myocardium of the early tube (section 1.2.3). Besides, the developing chambers do not derive from the entire heart tube myocardium (van den Berg and Moorman, 2009). The old idea that the chambers differentiated as circumferential segment along the length of the heart tube has been replaced by the *ballooning model* where the cardiac chambers balloon out from the outer curvature of the looping heart (fig. 1.5 B) (Moorman and Christoffels, 2003; van den Berg and Moorman, 2009). The formation of the chamber in the outer curvature of the looped heart is initiated by a high regional activation of increase in cell volume followed by an increase in the rate of proliferation of the large cells (Soufan *et al.*, 2006).

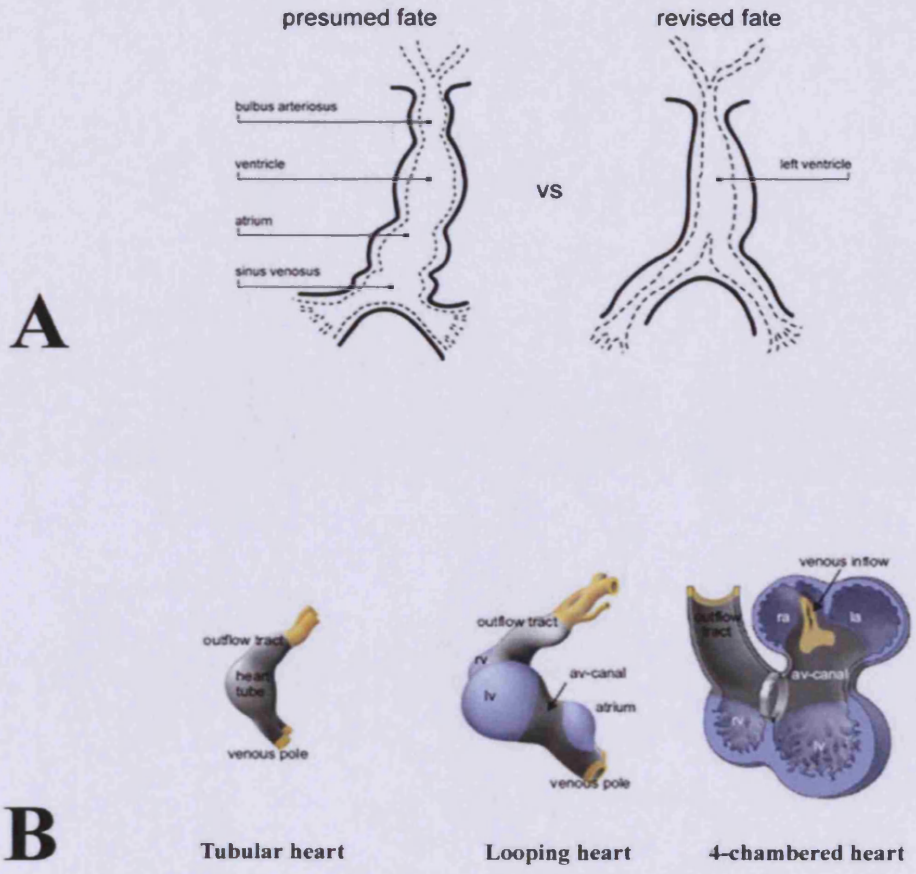

Figure 1.5 Mouse heart development (later view). Modified from den Berg and Moorman, 2009 and Boogerd *et al.*, 2009. (A) Recent researches have changed the original view of cardiac development where the heart tube contained all prospective cardiac chambers as circumferential segments. Recently has been confirmed that in the heart tube are present only the precursor cells of the left ventricle and the remaining myocardium is added during subsequent development. (B). Heart tube, generated from the fusion of the cardiac crescent in the ventral midline, is composed of inflow and outflow tract. During the heart looping the heart undergo to a rightward spiral movement where the ventricular component of the heart has moved to the right and balloon out from the outer curvature. The looping movement is therefore finally resolved with the formation of the 4-chambered heart. av-canal; atrioventricular canal, la; left atrium, lv; left ventricle, ra; right atrium, rv; right ventricle (Boogerd *et al.*, 2009; van den Berg and Moorman, 2009).

These regional changes in both size and proliferation occur prior to trabeculation and may suggest that the pathway of signals that control cell-size are also important for the regulation of the myocyte proliferation and differentiation (van den Berg and Moorman, 2009). The contractile apparatus of the future chambers develops in the outer curvature which shows, during the myocardium proliferation, an increase in conduction activity. In contrast, the myocardium of the inner curvature, the atrioventricular canal and the OFT remains poorly differentiated (van den Berg and Moorman, 2009).

Heart structure varies among vertebrates. After heart looping, the heart has two chambers in fish in which the atrium is directly connected with the ventricle (fig. 1.6). The evolution from aquatic to terrestrial environment has determined the developing of a complex heart structure which required the separation of oxygenated from deoxygenated blood. Moreover, the high volume-low pressure cardiovascular system present in tunicates (blood volume is equal 40% of the body weight) has evolved into a low volume-high pressure cardiovascular system in vertebrates (blood volume is equal 6% of the body weight). This change has driven the contracting straight tubular heart to transform into the more efficient and powerful synchronously contracting looped heart in vertebrates (Moorman and Christoffels, 2003). Amphibians heart is composed of two atria and one ventricle while terrestrial species (birds, reptile and mammals) show heart composed of two atria and two ventricle in which septae separate completely the oxygenated from deoxygenated blood in the pulmonary and systemic circulation (fig. 1.6) (Fishman and Olson, 1997; Olson, 2006).

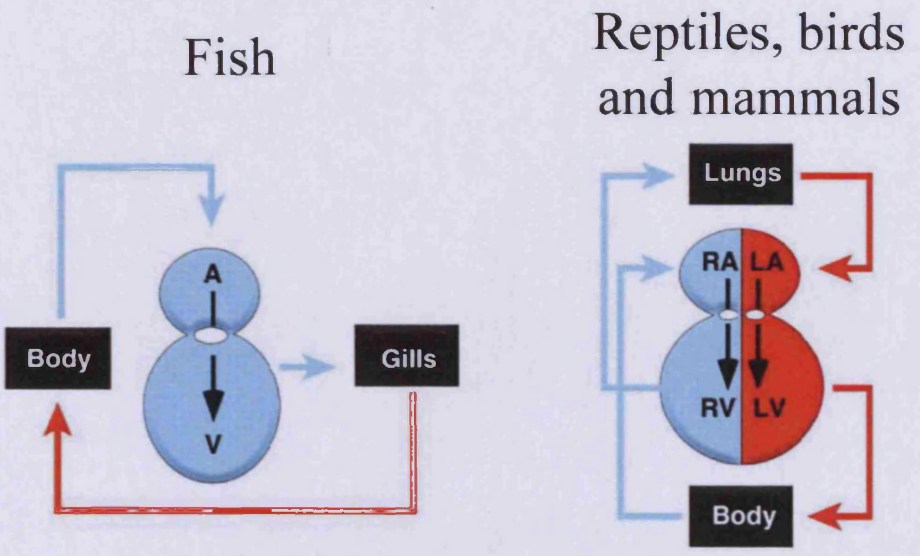

Figure 1.6 Anatomy of the heart in vertebrates. Modified from Olson, 2006. The heart in fish is composed of one atrium and one ventricle chamber while terrestrial vertebrates have the heart composed by two atrial and two ventricular chambers. Frog heart is evolutionary positioned between aquatic and terrestrial vertebrates heart. Amphibian heart is composed of two atria and one ventricle. Oxygenated blood is showed in red and deoxygenated blood is showed in blue (Olson, 2006).

1.2.2.1 Gene regulatory network during cardiogenesis

Cardiac development is regulated by evolutionary conserved transcription factors that coordinate the specification and differentiation of myocytes. These transcription factors (Nkx2.5, MEF2, GATA-4 and Tbx) respond to inductive signals in myocardial development and control the cardiac cells fate, the expression of genes encoding contractile proteins and the morphogenesis of cardiac structures (Olson, 2006). The Drosophila *tinman* vertebrate homolog Nkx2.5 is homeobox transcription factor. In the mouse Nkx2.5 expression is first observed in the precardiac mesoderm and in the pharyngeal endoderm (E7.5) and its expression continues at high level in the heart through adulthood (Akazawa and Komuro, 2005; Jamali *et al.*, 2001b). In Nkx2.5 null mice, the heart tube fails to undergo looping but the expression of the main cardiac-specific genes still occurs (Jamali *et al.*, 2001b; Lyons *et al.*, 1995). However, ventricular specific marker MLC2v is not expressed while myosin-heavy chain β and cyclin D2 show normal expression. This indicates that not all ventricle-specific genes are regulated by Nkx2.5 (Lyons *et al.*, 1995).

Nkx2.5 has been isolated in *Xenopus* as well. Its expression domain is similar to Nkx2.5 expression in mouse and *Drosophila* (Tonissen *et al.*, 1994). Nkx2.5 expression starts at gastrulation and persist at neurula stages where is present in the presumptive heart mesoderm. In the adult heart and later in the myocardium NKx2.5 expression starts to decrease (Tonissen *et al.*, 1994). It has been shown that Nkx2.5 synergistically interact with GATA-4 to activate transcription of cardiac genes such as ANF (Durocher *et al.*, 1997). It has been shown that the synergistic interaction between GATA-4 and Nkx2.5 regulates the expression of ANF specifically in the atrium (Small and Krieg, 2003).

The GATA zinc-finger transcription factor family is comprised of six members, but only three (GATA-4, -5, -6) have been shown to be important for heart development. In particular, GATA-4 is present in the gut, the gonads, the liver, the visceral endoderm and the parietal endoderm (Arceci *et al.*, 1993; Peterkin *et al.*, 2005; Pikkarainen *et al.*, 2004). In the mouse, GATA-4 mRNA is present at early developmental stages (E7.0) in the precardiac mesoderm and continues to be expressed during heart tube formation in the myocardium and endocardium (Kelley *et al.*, 1993). During heart morphogenesis GATA-4 is also required for the proper assembly of the heart tube formation (Kuo *et al.*, 1997).

Mef2 (myocyte enhancer factor 2) family of transcription factors comprises four members (Mef2-A, -B, -C, -D) which bind a conserved A-T rich DNA sequence. Mef2 family members are expressed in cardiogenic precursor cells and in differentiated cardiomyocytes (Lin *et al.*, 1997). Mef2C regulates cardiac myogenesis and the right ventricular development. It is expressed in the cardiac precursors before the heart tube formation and is required for the activation of cardiac contractile protein. In Mef2C null mice the heart fails to undergo looping and lacks the right ventricle chamber. The ablation of Mef2C prevents the selective activation of specific cardiogenic transcription factors such as HAND2 and HAND1. HAND2 transcripts were downregulated during the heart looping concomitant with failure of the right ventricular region to form. HAND1 expression in the mutants was contiguous throughout the heart tube without the gap in expression that normally characterises the future right ventricle. Distortion in HAND1 expression and the lack of HAND2 expression in the heart tube are consistent with the lacking of right ventricle in mutant embryos (Lin *et al.*, 1997).

T-box genes family is composed, in vertebrate, of at least 18 members which together play important roles in cardiac lineage determination, chamber specification, valve formation, epicardial development and formation of the conduction system (Plageman and Yutzey, 2005). Mutations of T-box transcription factors lead to congenital heart disease as atrial septal defect in Holt-Oram syndrome (Tbx5 mutation) (Basson *et al.*, 1997) and cardiac outflow tract and pharyngeal arch abnormalities in DiGeorge or 22q11 deletion syndrome (Tbx1 mutation) (Baldini, 2004). Thus, T-box family show an important role as regulator of the heart development. For example the interaction between Tbx5, Tbx20, Nkx2.5 and GATA-4 lead the atrial chamber differentiation through the regulation of ANF which is suppressed in the atrioventricular canal from Tbx2 and Tbx3 expression (Hoogaars *et al.*, 2007). Tbx1 is specifically expressed in the second heart field (SHF, section 1.2.3) and provides extensive contribution to the OFT via the regulation of forkhead (Fox) transcription factors. Tbx20 is expressed in the primary heart field (PHF) and plays an important role in the recruitment and elongation of the heart tube (Hoogaars *et al.*, 2007). Tbx18 is expressed in a small subpopulation of cells located ventrally to the heart tube which gives rise to the proepicardium (Jahr *et al.*, 2008). In *Xenopus*, Tbx18 is specifically expressed from stage 36 in the right horn of the sinus venosus and no expression has been found in the left sinus horn (Jahr *et al.*, 2008). Nkx-2.5 and Isl-1expressing cells contribute to regulate the formation of proepicardium (Zhou *et al.*, 2008) suggesting a functional role for Nkx2.5 in proepicardium formation.

The conserved core of transcription factors regulates the specification of two distinct progenitor cell populations known as primary and secondary heart field. In amniotes, the primary heart field (PHF) forms the left ventricle and portions of the atria, while the secondary heart field generates the right ventricle, portions of the atria and outflow tract (Laugwitz *et al.*, 2008).

1.2.3 Heart field formation

The heart is generated from muscle and non-muscle cell lineages, including cardiac myocytes, conduction system cells, smooth muscle and endothelia cells (Laugwitz *et al.*, 2008). The differentiation of these multiple tissue type is regulated under specific temporal and spatial coordinated control in which different tissues become components of the developed heart. How these different tissues arise during heart development is a question still under investigation. Three major sources of cardiac precursors have been identified in mouse: cardiogenic mesoderm, cardiac neural crest and proepicardial organs (Laugwitz *et al.*, 2008). Cardiogenic mesoderm gives rise to the heart tube from which atrial and ventricle chambers are formed (fig. 1.7 B). Cardiac neural crest migrates to the heart after heart looping to generate the vasculature smooth muscles of the aortic arch and ductus arteriosus (fig. 1.7 B). the proepicardium generates heart mesenchyme and the epicardial cells (fig. 1.7 B) (Jahr *et al.*, 2008). Different studies demonstrate that cardiogenic mesoderm consist of two distinct fields of cells which contribute to different regions of the heart. It is known that a source of cells identified as PHF is generated from cardiac crescent to develop the heart tube containing the outline of left ventricle, atrio-ventricle structure and atria (fig. 1.8). The origin of this field has been extensively investigated because it was not clear where the outflow tract myocardium (OFT) originated from. The question of which progenitor cells contribute to the OFT has been unresolved for many years in part because experiments were performed in explanted embryos which die before the OFT was completed.

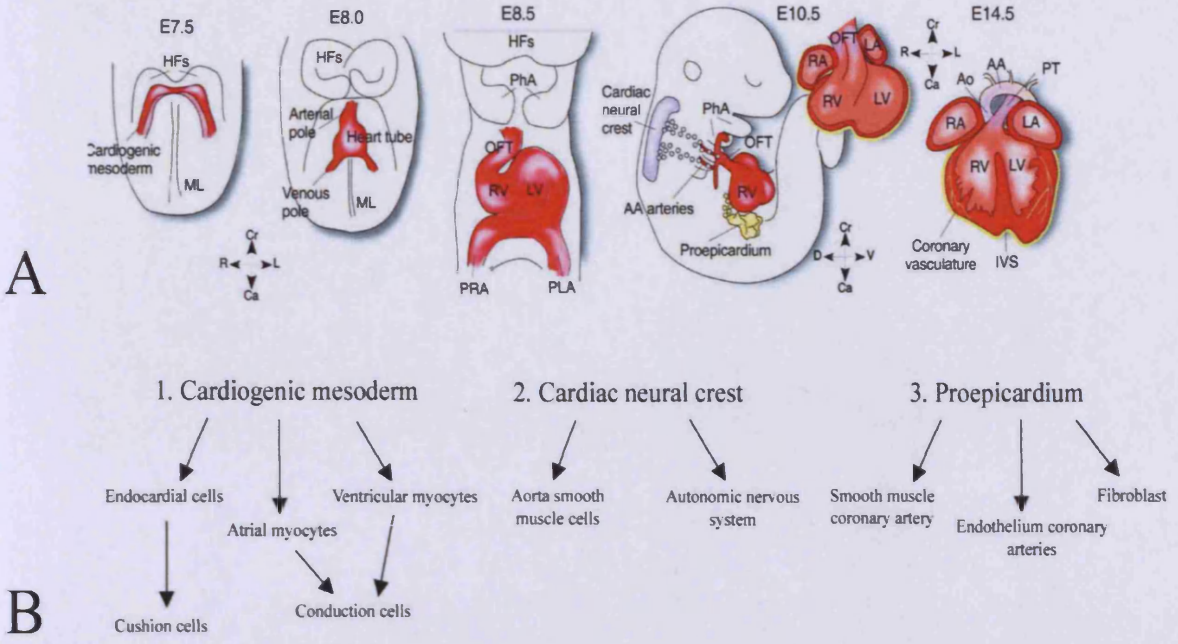

Figure 1.7 Different cardiac cell types in heart development. Modified from Laugwitz *et al.*, 2008. (A) Heart development starts from cardiac crescent which lately generates the heart tube. Heart looping involves anticlockwise spiral movement resolved with the formation of a chambered heart. (B) Three population of embryonic heart precursor contribute to the heart development. Cardiogenic mesoderm gives rise mainly to the atria and ventricular myocytes, cardiac neural crest gives rise to aorta smooth muscles and proepicardium gives rise to endothelium. Cranial (Cr)-caudal (Ca), right (R)-left (L), and dorsal (D)-ventral (V) axes are indicated. AA, aortic arch; HFs, head folds; IVS, interventricular septum; LA, left atrium; LV, left ventricle; ML, midline; PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; RA, right atrium; RV, right ventricle (Laugwitz *et al.*, 2008).

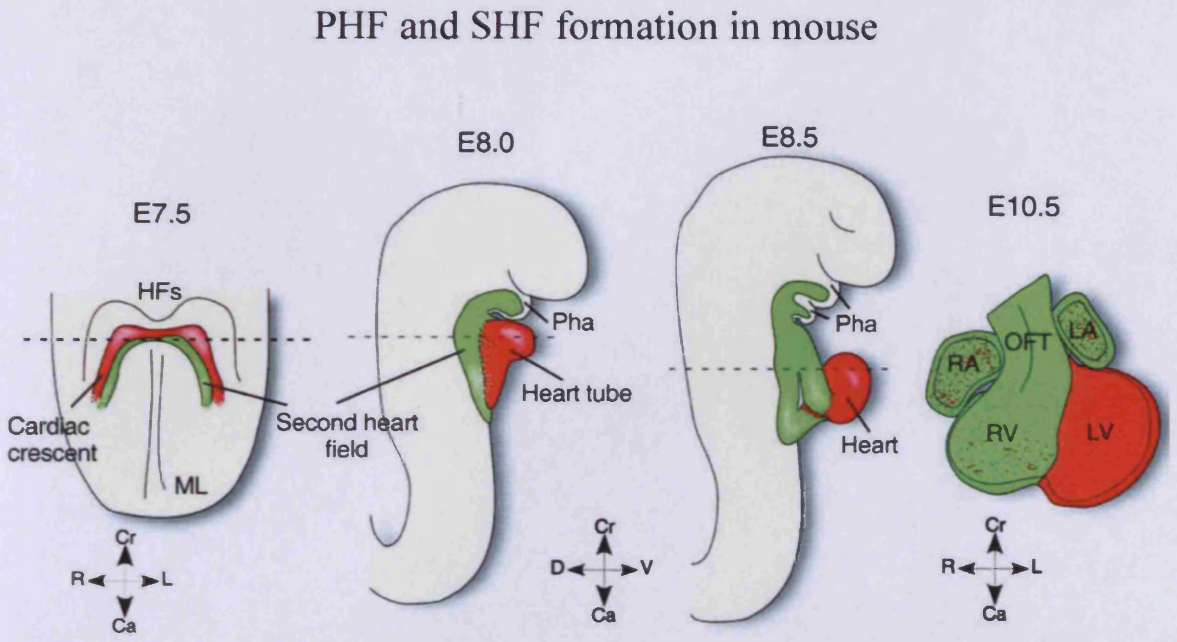

Figure 1.8 Primary and Second heart field in mouse and chick. Taken from Laugwitz et al., 2008. In mouse heart development PHF (showed in red) contributes to generate the left ventricle and portion of the atria, while the SHF (showed in green) contribute mainly to generate OFT, right ventricle and portion of the atria. Cranial (Cr)-caudal (Ca), right (R)-left (L), and dorsal (D)-ventral (V) axes are indicated. LA, left atrium; LV, left ventricle; ML, midline; PhA, pharyngeal arches; OFT, outflow tract; RA, right atrium; RV, right ventricle (Laugwitz *et al.*, 2008).

Initial evidence of distinct cells contributing to the OFT formation came from *in vivo* labelling experiments in chick embryos in which it was shown that the cranial-most outflow region was formed by a secondary source of myocardium progenitors (de la Cruz *et al.*, 1977; Dyer and Kirby, 2009). More recently three different studies demonstrate the existence of a population of cells that contribute to the heart formation after the heart tube has formed. In particular Kelly *et* colleagues, showed through the use of FGF10-nlacZ reporter in mouse that heart is formed from two sources of myocardial precursors which respectively give rise to the heart tube and a second population of FGF10 positive cells in the pharyngeal mesoderm where the OFT and possibly the right ventricle form (Dyer and Kirby, 2009; Kelly *et al.*, 2001; Laugwitz *et al.*, 2008). Labelling studies performed in chick embryo demonstrate that splanchnic mesoderm migrate to contribute to the OFT and the right ventricle cell population generating a field of population called anterior heart field or secondary heart field (Dyer and Kirby, 2009; Laugwitz *et al.*, 2008; Mjaatvedt *et al.*, 2001; Waldo *et al.*, 2001). The precursors cells that give rise to the OFT and right ventricle has been variously described as second heart field, anterior heart field or secondary heart field. The lack of a proper nomenclature has generated controversy but a consensus is emerging that what is referred to as anterior and secondary heart field is considered to be a subpopulation of the second heart field (Perez-Pomares *et al.*, 2009) which give rise to OFT and the right ventricle. However, the confusion in defining this field of cells may originate from the use of different techniques in a variety of different organism. For this reason the most reliable methods to define the second heart field population is via gene expression analysis of specific markers.

1.2.3.1 Gene expression in the SHF

The LIM-homeodomain transcription factor Isl-1 has been identified as a marker of the second heart field. Lineage analysis showed that Isl-1 expression is present in the right ventricle, OFT and atria (Cai *et al.*, 2003). This analysis was confirmed in Isl-1 null mice which completely lack the OFT and right ventricle. Morevoer, Isl-1 mutants show reduced Isl-1 expression in progenitor cells with a downregulation of FGF and BMP growth factors (Cai *et al.*, 2003). Isl-1 positive progenitor cells generate a distinct second myocardial lineage which supplements the myocardium derived from first heart field progenitors. Right ventricle, OFT and part of the atrial cells express Isl-1 and proliferate prior to the onset of cardiac differentiation after when Isl-1 is downregulated (Cai *et al.*, 2003; Laugwitz *et al.*, 2008; Moretti *et al.*, 2007). It has been shown that Isl-1 and Nkx2.5 mark a cell population which gives rise to the myocardial cells, a subset of endocardium and aortic endothelium (Cai *et al.*, 2003; Moretti *et al.*, 2006). The possibility that Isl-1 marks multipotent primordial cardiovascular progenitors which gives rise to distinct cell lineages within the second heart field was confirmed by Moretti and colleagues using genetic fate-mapping in mice. The primordial multipotent cardiovascular progenitor cells positive to Isl-1 can generate different cell types (endothelial, myocardium and smooth muscle) during *in vivo* embryonic development (Moretti *et al.*, 2006). This demonstrates that the multipotent Isl-1/Nkx2.5 progenitor expressing cells represent a common cardiac progenitor which contributes substantially to the embryonic heart. Recent studies carried out using both Nkx2.5 and Isl-1 Cre mice showed that both of these genes have a similar expression domain throughout the heart field of the lateral plate mesoderm demonstrating that the differences between the primary and the second heart field progenitors depend entirely on the timing of differentiation during heart development (Dyer and Kirby,

2009; Ma *et al.*, 2008). This is supported by evidence in *Xenopus* where Isl-1 and Nkx2.5 are co-expressed in the cardiac crescent during neurula stages (Brade *et al.*, 2007). Other studies have shown that Isl-1 is partially expressed in the left ventricle (PHF) (Cai *et al.*, 2003; Moretti *et al.*, 2006; Prall *et al.*, 2007). Thus, the initial hypothesis that Isl-1 could be used as exclusive marker of the second heart field has been revised. The current hypothesis suggests that Isl-1 marks a broad range of undifferentiated progenitor cardiac cells which mainly contribute to the secondary heart field. It has been suggested that the role of Isl-1 as pan-cardiac progenitor cell marker mainly expressed in the SHF (Laugwitz *et al.*, 2008; Prall *et al.*, 2007). Thus, the first and the second myocardial lineages seems to be regulated by the same core of cardiac transcription factors (GATA, Nk, SRF) which are expressed under the control of BMP and FGF pathways (Buckingham *et al.*, 2005). In particular, in the SHF the forkhead box H1 DNA binding transcription factor Foxh1 is directly expressed under the regulation of Isl-1. Foxh1 regulates the expression of Mef2c in collaboration with Isl-1, GATA factor and Nkx2.5 (Dodou *et al.*, 2004; Laugwitz *et al.*, 2008; Perez-Pomares *et al.*, 2009; von Both *et al.*, 2004). In the PHF the expression of downstream targets Tbx5 and Hand1 are regulated by the same core of transcription factor including GATA family, Nkx2.5 and SRF (Black, 2007; Cai *et al.*, 2003; Perez-Pomares *et al.*, 2009). The main differences between the two fields depends entirely on differential regulation of the same transcriptional network (Perez-Pomares *et al.*, 2009) and different spatiotemporal contribution to the heart. Nkx2.5 is the main regulator of the PHF and Isl-1 whereas GATA4 and Foxh1 are the key transcription regulator in the SHF (Laugwitz *et al.*, 2008).

1.2.3.2 Regulation of the SHF by signalling pathways

The development and proliferation of the SHF during heart tube elongation has been shown to be regulated by a set of signaling pathways including Wnt, FGF, BMP and retinoic acid (RA). The canonical Wnt pathway showed an important role in the regulation of SHF proliferation. It regulates the renewal and differentiation of Isl-1 positive cardiac progenitors (Qyang *et al.*, 2007) and also plays an important role in promoting the expansion of Isl-1 positive cells through the activation of FGF ligands (Cohen *et al.*, 2007). Moreover, the canonical Wnt pathway promotes growth and diversification of SHF precursors into right ventricle and interventricular myocardium (Ai *et al.*, 2007; Rochais *et al.*, 2009).

FGF10 was the first gene identified in the SHF (Kelly *et al.*, 2001). FGF8 loss in mouse was shown to be fundamental in the proliferation and survival of the SHF and requirement in the OFT septation (Ilagan *et al.*, 2006; Park *et al.*, 2006; Rochais *et al.*, 2009). Moreover, Ilagan and colleagues speculated that FGF8 signal increases the cell motility in order to allow the cells belonging to the SHF to migrate into the heart tube (Ilagan *et al.*, 2006).

Evidence reveals an important role for BMP during the SHF differentiation and development. OFT septation and development of the smooth muscle and endocardial cushion are under the control of BMP4, while BMP2 recruits SHF cells to develop the arterial pole. Also, the elongation of the OFT is regulated by BMP4 and 7 (Rochais *et al.*, 2009).

Retinoic acid (RA) has been shown to be involved in the SHF development. The lack of retinaldehyde dehydrogenase (Raldh2), enzyme involved in the RA synthesis, leads to a strong

posteriorisation of SHF markers (Isl-1, FGF10, FGF8). In fact, it has been demonstrated that RA restricts the number of cardiac progenitor cells in zebrafish (Keegan *et al.*, 2005) and is required to limit the expansion of Isl-1 positive cells posteriorly through the reduction of FGF8 expression (Sirbu *et al.*, 2008).

1.2.4 Axial patterning of the heart

The development of a complex multichambered heart structure is dependent on the correct establishment of the anteriorposterior (A-P) and left-right (L-R) polarity in the heart tube. The polarity is established through the regional cardiac cell diversification along the A-P axis and is resolved with the specification of the atrial and ventricle primordia in a defined anterior and posterior order. This determines the correct progression of cardiac morphogenetic events from heart tube looping to chamber formation. It is known that heart pattern establishment through the A-P and L-R axis is directed by a number of signals such as retinoic acid (RA), Nodal, FGF and BMP.

1.2.4.1 Retinoic Acid

Retinoic acid (RA) is the active form of vitamin A. It is formed by two oxidative reactions in which retinaldehyde is synthesised from retinol and subsequently retinoic acid is synthesised from retinaldehyde. These reactions are catalysed by two different enzymes; alcohol dehydrogenase (ADHs) and retinaldehyde dehydrogenase (RALDH2) (Hochgreb *et al.*, 2003).

RA, after synthesis, binds RA nuclear receptors (RARα, β, and γ) and retinoid X receptor (RXRα, β, and γ). This complex works as a ligand-activated transcription factor, which transmits the signal for the activation of genes that contain the retinoic acid response elements (RAREs) (Collop *et al.*, 2006).

Anterior-posterior polarity is one of the most important decisions during heart development and this event happens prior to the synthesis of contractile proteins in vertebrates cardiac cells (Xavier-Neto *et al.*, 2001). Multiple studies have confirmed the role of RA in the specification of the anterior-posterior polarity in the heart. Excess RA induces a dose- and stage-dependent expansion of atrial compartment and consequently a at the expense of the ventricular tissue (Xavier-Neto *et al.*, 2001). Chicken embryos treated with RA and analysed by the expression of atrial myosin heavy chain 1 (AMHC1) and ventricular myosin heavy chain 1 (VMHC1) show strong atrialisation of the heart with concomitant expansion of AMHC1 expression domains (Yutzey *et al.*, 1995). Quail embryos deficient in RA have an oversized ventricle and lack atrial structures and ophalomesenteric veins (Heine *et al.*, 1985; Xavier-Neto *et al.*, 2001). It has been shown in chick that cardiomyocytes adopt regional identity along the A-P axis soon after gastrulation and the cardiac diversification can be altered throughout RA treatment before differentiation. Cardiogenic mesoderm identity is definitively established just before contractile protein gene expression and it can no longer be altered by RA treatment after differentiation occurs (Yutzey *et al.*, 1995). These and other studies with retinaldehyde dehydrogenase (RALDH2) enzyme, which is involved in the synthesis of RA, support the model that during heart development RA signaling induces posterior (future sinoatrial) cell fate, while the specification of the anterior fate (future ventricules and conotruncus) is induced in absence of RA (Xavier-Neto *et al.*, 2001). Studies performed in the chick embryo analysing RALDH2 expression indicate that heart patterning is established in two distinct phases.

During the first phase, called specification phase (developmental stage HH5-7), there is an increase of RALDH2 expression in the posterior mesoderm close to the sino-atrial precursor. In the second phase, called determination phase (developmental stage HH7-8), there is a strong expression of RALDH2 which encircles the sino-atrial precursor generating a wave of expression from the caudal to the rostral region of the lateral plate mesoderm (Hochgreb *et al.*, 2003). This demonstrates that the A-P axis specification from RA is provided by the posterior mesoderm. Studies on zebrafish embryos have demonstrated that RA affects heart patterning differently during different developmental stages. Pan-retinoic acid receptor (RAR) antagonist experiments have established that RA signaling is required during gastrulation. When RAR antagonist is added before gastrulation, there is a consistent myocardial expansion indicating that RA restricts the cardiac progenitor pool during cardiac specification creating a balance between cardiac and non-cardiac identity. However, if RA antagonists are added in zebrafish after gastrulation the number of cardiomyocytes remained the same (Keegan *et al.*, 2005). Thus, RA is required before gastrulation to regulate the cardiomyocytes population size. *Xenopus* embryos which are treated with RA antagonist before gastrulation result in overexpression of Nkx2.5 which is consistent with the myocardial expansion phenotype described in zebrafish (Collop *et al.*, 2006; Keegan *et al.*, 2005) suggesting that RA maintains the balance between cardiac and non cardiac cell identity ensuring that appropriate heart size develops (Keegan *et al.*, 2005). Similar hypothesis has been shown by Drysdale and colleagues. If embryos are treated with RA after cardiac specification there is an inhibition of myocardial differentiation markers, but the endocardium does not appear to be affected. RA treatment after heart differentiation does not show any significant changes. Myocardial differentiation is sensitive to RA expression which restricts heart field growth during cardiogenesis (Drysdale *et al.*, 1997). However, Collop and colleagues have demonstrated that

Xenopus embryos treated with RA antagonist after gastrulation show a failure in the formation of the heart tube. In experiments with lower concentration of RA antagonist, cardiac development continues but is blocked before the normal morphogenesis is completed (Collop *et al.*, 2006). Thus, RA is involved in different stages during heart development in order to control the correct proportion of cardiomyocites to generate the final heart structure. It has also been established that RA plays an important role in the formation of left and right asymmetry. *Xenopus* embryos treated with a RA antagonist at stage 32-34 and examined at later stages presented abnormal hearts with "some left-right asymmetry", but lacking a vascular system connection and circulating blood (Collop *et al.*, 2006). It has been shown that RA controls the expression of *lefty*, *Pitx2* and *nodal* during L-R patterning. RA treatments show a randomisation of looping and symmetrical expression of *lefty*, *nodal* and *Pitx2*. Therefore, RA may synergise with the expression of the left-restricted factors (Chazaud *et al.*, 1999).

1.2.4.2 Nodal

During vertebrate embryo development Nodal acts as inducer of mesendoderm. It also regulates embryonic axis establishment, nervous system patterning and the left and right asymmetry (Schier, 2003). Baker and colleagues, showed a distinct direct and indirect role of Nodal during the asymmetric cardiac morphogenesis in zebrafish (Baker *et al.*, 2008). Nodal directly influences the conversion of cardiac cone to heart tube (cardiac jogging) whereby the atrial cells are repositioned to the anterior-left of the ventricle cells. The cardiac cone rotates and changes the original L-R axis to the D-V axis of the linear heart tube. The second asymmetry during cardiac looping is not influenced directly by Nodal. Cardiac looping

rotation establishes ventricle position to the right of the atrium and is directly dependent on the direction of the jogging (Baker *et al.*, 2008). Moreover, it has been shown that Nodal has a direct role in the establishment of the site of inner ventricular and outer atria curvature (Baker *et al.*, 2008). Evidence in zebrafish showed that Nodal regulates left-right asymmetry during heart morphogenesis by controlling the speed and direction of cardiomyocites movement. BMP asymmetric expression is dependent on regulation by asymmetrically expressed Nodal and both regulate the asymmetric behaviour of cardiomyocite movement during heart development (de Campos-Baptista *et al.*, 2008).

Nodal and *lefty* are expressed in the left lateral plate mesoderm (LPM) where Nodal plays an important role in the initial asymmetry of the anterior LPM. However, it has been shown in *Xenopus* that these signals are only transiently expressed, while the homeobox gene *Pitx2c* continues to be expressed during the looping of the heart and the gut (Campione *et al.*, 1999). The Nodal target Pitx2c is asymmetrically expressed in the LPM and the expression domain is conserved in amphibians, fish and mammals (Campione *et al.*, 1999). *Pitx2c* is expressed from stage 26 in *Xenopus* and is clearly involved in the regulation of the looping movement in the heart and in the gut (Campione *et al.*, 1999).

1.2.4.3 FGF

Evidence from chick embryos suggest that FGF8 is expressed in the right side of the Hensen's node and represses the left expression of Nodal and Pitx2c as a right determinant (Boettger *et al.*, 1999). Moreover, it has been shown that FGF8 has a direct involvement in the establishment of looping direction during heart development. Left-sided application of FGF8 in the Hensen's node showed a randomisation of the direction of the heart situs with a

predominant left-sided looping phenotype (Boettger *et al.*, 1999). Similar evidence was demonstrated in mice where Fgf8 mutants display abnormal looping to the left side when analysed at embryonic day 9.5 (Meyers and Martin, 1999). Moreover, Meyers and colleagues have shown more detailed cardiovascular role for FGF8 during heart development. FGF8 mutant mice exhibit strong cardiovascular defects when analysed at E16.5 including a lack of OFT septation, abnormal septation with double outlet right ventricle (Tetralogy of Fallot), abnormal development of aortic arch arteries and aberrant vasculature structure (Abu-Issa *et al.*, 2002). Work in zebrafish showed a specific requirement of Fgf8 during heart development. Fgf8 mutant (*ace*) embryos show a distinct defect in the ventricle chamber which is greatly reduced in size. This evidence highlights the possibility that Fgf8 is required for induction and patterning of myocardial precursors acting upstream of cardiogenic transcription factors Nkx2.5 and GATA-4 (Reifers *et al.*, 2000).

1.2.4.4 BMP

Evidence suggests that BMP acts to regulate the L-R axis establishment. In particular BMP4 has been shown to play an important role in heart looping morphogenesis via the expression of the *Xenopus* nodal related protein Xnr1. BMP4 is expressed in the left side of the heart tube in *Xenopus* and zebrafish from the heart tube stage until the onset of the heart looping (Breckenridge *et al.*, 2001). BMP affects the L-R axis but does not interfere with the regional specification of the chambers along the A-P body axis. Moreover, Xnr-1 acts upstream of BMP4 and induces the asymmetric left-sided expression of BMP4 in the heart tube. In fact, ectopic expression of Xnr-1 affects the asymmetric expression of BMP4 in the heart tube. It is possible that BMP4 expression is mediated by upstream molecules such as lefty and Pitx2c

because Nodal expression in the LPM ends before the heart tube has been generated (Breckenridge *et al.*, 2001). Similar evidence in zebrafish showed that BMP-4 is expressed in the left side of the heart during the jogging stage when the midline of the heart "jog" to the left and loops to the right (22-somite stage). BMP4 expression is linked with the direction of the jogging and looping of the heart (Chen *et al.*, 1997). If BMP4 expression is symmetric the heart does not jog and looping does not occur (Chen *et al.*, 1997). Recent evidence in zebrafish showed that BMP signalling is required to regulate the migration of cardiac progenitor cells and rotation during heart looping (Smith *et al.*, 2008) in order to generate asymmetric organ development. This evidence agrees with the role of BMP4 in directing the jogging and the looping of the future asymmetric heart morphogenesis along the L-R axis (Smith *et al.*, 2008). Rotation of the heart requires Has2 which controls the right migration of the cardiac precursors and is finally directed by BMP towards the LPM. Coordination of these genes regulate the heart tube formation and the future pattering of the heart (Smith *et al.*, 2008).

1.3 Heart development of Xenopus laevis

1.3.1 Amphibian as a model for vertebrate cardiogenesis

Study of the heart development in mammalian embryos is limited by early lethality of cardiac malformations. Heart development is sensitive to genetic perturbations. The limitations in using mouse as a model for study heart development can be overcome by the use of *Xenopus* embryos. *Xenopus* heart development has been clearly elucidated from cardiac induction to chamber formation. Frog heart is composed of three chambers, one ventricle and two atria. For this reason it is evolutionally located between the two-chambered fish heart and the four-chambered amniote heart (Warkman and Krieg, 2007). These morphological differences are not restrictive because heart development is governed by a core of transcription factors evolutionally conserved between vertebrate and invertebrate species (Olson, 2006). One major advantage to use *Xenopus* to study the heart development is its lack of dependence on cardiovascular system (Blitz *et al.*, 2006). Thus, any gene manipulation concerning the heart development can be studied at all developmental stages as they are not immediately lethal for the embryos. Therefore, *Xenopus* is highly suitable to study the mechanisms of organogenesis in whole embryo and in undifferentiated cells as in the case of *in vitro* organogenesis.

1.3.2 Heart morphogenesis in Xenopus laevis embryos

Heart mesoderm specification occurs during gastrulation (Sater and Jacobson, 1989) when the cardiac progenitors arise from the lateral periphery of both lateral sides of the embryo. During neurula stage they move dorsally and laterally to fuse on the ventral midline to form a delta-

shaped region (fig. 1.9, stage 27) (Kolker *et al.*, 2000). At this stage the heart field expresses pan-myocardial markers MLC2 (Latinkic *et al.*, 2004), Nkx2.5 (Tonissen *et al.*, 1994) and MHC-α (Logan and Mohun, 1993; Mohun *et al.*, 2000) and is located along the anteroventral region behind the cement gland (Mohun *et al.*, 2000). The expression of MLC2 and MHC-α indicate the onset of cardiac differentiation where the future heart is orientated along the A-P axis (Mohun *et al.*, 2000). At stage 29-30 the splanchnic mesoderm (formed from anterior mesoderm) is concentrated in the heart-forming region and develops as a ventral trough formed from myocardial tissue. The endocardial tube, which starts to develop from the anterior end of the splanchninc mesoderm, is encompassed by the myocardial ventral trough. At the same time, the somatic mesoderm, consisting of a single cell layer, localises under the ventral ectoderm to form the ventral and lateral walls of the pericardial cavity (Mohun *et al.*, 2000). At stage 31-32 (fig. 1.9) the myocardial trough surrounds the dorsal endocardial tissue to generate the heart tube. The anterior end of the myocardial trough does not completely surround the endocardial tissue, which bulges to generate a sinus (aortic sac). This develops into the ventral aorta and bilaterally into the left and right first aortic arch. At the posterior end the endocardium generate the sinus venosus which bifurcates and extends caudally towards the liver primordium. The myocardium encompasses the sinus venosus dorsally and laterally as far as the bifurcation point. The dorsal mesocardium, generated from the myocardium and the splanchnic mesoderm, extends posteriorly to generate the major vessels of the venous system. At this point the linear heart tube is completed and retains this linear structure for several hours until heart looping occurs.

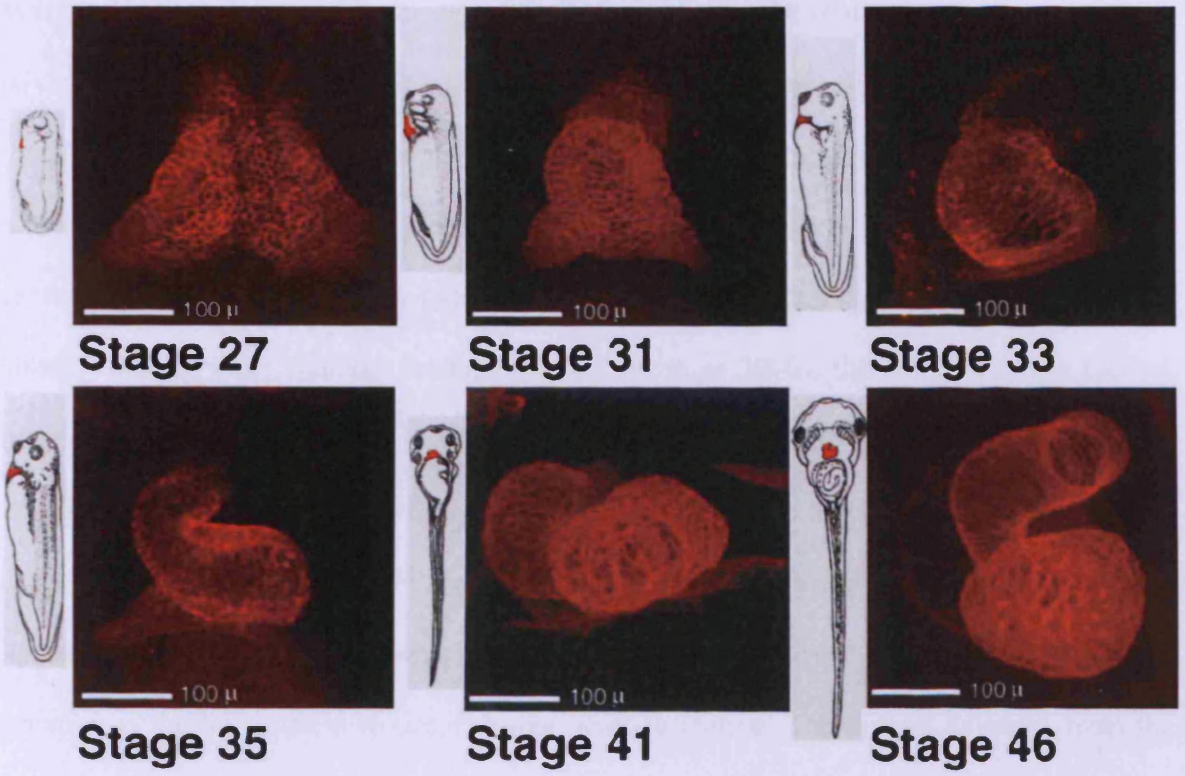

Figure 1.9 Major steps of *Xenopus* heart development. Taken from Kolker *et al.*, 2000. Heart development is described throughout the major developmental steps in which the fusion in the ventral midline of the cardiac precursor generate the heart tube (stage 31). At stage 33-35 the heart tube loops in a rightward spiral movement in which the final heart structure is completed with the formation of one ventricle and two atrial chambers (stage 46) (Kolker *et al.*, 2000).

At stage 35 (Fig.1.9, stage 33 and stage 35) the linear heart tube changes its anterior-posterior axis direction because the posterior side (presumptive atrium and sinus venous) moves dorsally, more closely to the anterior region and the heart tube moves anticlockwise in a spiral motion in the process known as heart looping. Consequently, the OFT (positioned anteriorly) moves medially and the conus (positioned posteriorly) relocates to the right side of the embryo. During heart chamber formation (fig. 1.9, stage 39-40) the looped heart is located along the A-P axis. The atrium is located dorsally compared to the ventricle and at the same time the development of trabeculae (generated from ventriculae myocardia) and valve occurs. The atrioventricular valve and valves into the OFT will be clearly resolved at stage 45-46 (Mohun *et al.*, 2000) when the heart chamber are completely formed (fig 1.9, stage 46). Atrial septation is the last process to occur during heart formation. The septum develops from the dorsal region of the atrium and divides the atrium in two distinct and unequal chambers with the right atrium being larger than the left. When septation is complete the left and right atria are connected respectively with the pulmonary vein and sinus venosus respectively. Therefore, at the end of tadpole development, the heart is composed of septated atria, a single atrio-ventricular valve, and one not septated ventricle which is highly trabeculated (Mohun *et al.*, 2000). Oxygenated and deoxygenated blood converges into the single ventricle from the left and right atrial chambers respectively. The incomplete outflow tract has two valves in series which direct the blood into either the pulmonary arteries or systemic arteries (Lohr and Yost, 2000).

1.3.2.1 Heart growth

Mouhun and colleagues compared the change in cell number during heart tube and looping movement. Nuclei counting in successive pairs of sections showed twice the number of myocardial cells in the central looped portion of the myocardium compared with linear heart tube. The most anterior and the most posterior regions of the looped heart show a smaller increase (Mohun *et al.*, 2000). Different evidence shows that heart size regulation is related to signals secreted from the dorsal side during gastrulation and prior to neural tube closure (Garriock and Drysdale, 2003). Embryos lacking all dorsal structures showed an increase of heart size with thicker myocardium and increased expression of GATA-4 and Nkx2.5 (Garriock and Drysdale, 2003). The heart field is spatially restricted during post-neurula stages (Sater and Jacobson, 1990a) and it corresponds with the domain of expression of Nkx2.5. Nkx2.5 is expressed also in the lateral portion of the heart field which is initially specified to form myocardium but later is respecified to become dorsal mesocardium and the roof of the epicardium (Raffin *et al.*, 2000). Thus, during the heart development a redirection of the cell fate occurs in which the mesocardium and pericardium are generated from the myocardium. The loss of heart-forming region is correlated with a decrease of Nkx2.5 and GATA-4 close to the dorsal tissue suggesting that heart restriction occurs prior the subdivision of the myogenic and non-myogenic compartments (Garriock and Drysdale, 2003). Another interesting aspect of heart development concerns the inverse relationship between the proliferation and differentiation of cardiac cells. Molecules that induce cell cycle exit promote cell differentiation. Movassagh and Philpott showed that cycline-dependent kinase inhibitor p27Xic is required for cardiac differentiation in *Xenopus* heart development (Movassagh and Philpott, 2008). Identification of molecules that regulate the differentiation or proliferation of

myocytes is crucial for understanding the regulation of cardiac differentiation. It has been shown that by stage 29-30 the majority of the cells have left the cell-cycle and only a small portion of differentiated cardiomyocytes still proliferate until stage 41. This demonstrates that cardiac differentiation occurs normally when cell cycle is inhibited. In fact, inhibition of the cell cycle from post-gastrula stage does not effect cardiac differentiation but lack of Xic leads to a substantial reduction of the differentiation markers with a consequent reduction of the heart size. It may be possible that Xic binds the transcription factors which lead cardiac differentiation to promote cardiogenesis (Movassagh and Philpott, 2008).

1.3.3 Heart tissue composition

Vertebrate heart is composed of tissues divided into three layers: epicardium, myocardium and endocardium. The epicardium is the outside layer and surrounds the myocardium tube. The endocardium is the innermost cell layer in the heart tube.

1.3.3.1 Epicardium

Epicardium originates from the proepicardium which develops in the posterior limits of the heart between the sinus venosus and the liver (Manner *et al.*, 2001). Epicardium is the outer layer of the vertebrate heart. Mesenchymal cells delaminate from epicardium and invade the myocardium and subepicardial spaces to provide the cardiac vessel and the connective tissue. Moreover, epicardium is involved in the differentiation of the embryonic myocardium (Manner *et al.*, 2001; Perez-Pomares *et al.*, 2009; Smith and Bader, 2007). The epicardium is

characterised by the expression of transcription factors as Wilm's tumor (WT-1), Pod-1/epicardin (Perez-Pomares *et al.*, 2009; Ratajska *et al.*, 2008) and Tbx18 (Cai *et al.*, 2008; Jahr *et al.*, 2008). It has been shown in zebrafish that BMP4 is required for the proepicardial specification via the expression of Tbx5 which confers to the anterior lateral plate mesoderm the capacity to respond to the BMP signal (Liu and Stainier, 2010).

1.3.3.2 Myocardium

Myocardial cells are believed to originate once endocardial progenitors delaminate from the polarized ephithelium which forms the visceral layer of the lateral plate mesoderm (LPM) (Perez-Pomares *et al.*, 2009). LPM is linked with the pharyngeal endoderm which is fundamental in the cardiac muscle development (Lough and Sugi, 2000). Myocardium can be divided into primitive myocardium and chamber myocardium (Perez-Pomares *et al.*, 2009). Primitive myocardium is located in the inflow tract, the AV region and the inner curvature of the heart tube. Unlike the chamber myocardium, the region which constitute the primitive myocardium show a low mitotic activity (Sedmera *et al.*, 2003) and is directly correlated with the development of the conduction system. The chamber myocardium corresponds to that of the heart tube and atrial and ventricle chambers. It has been shown that it is generated from a pool of rapidly proliferating cardiac precursor cells. The proliferation slows as they differentiate to cardiomyocytes but increase in size. The largest cells are found in the ventricle demonstrating that the increase in size of cardiomyocytes is chamber dependent (Soufan *et al.*, 2006). Chamber myocardium and primitive myocardium are regulated by T-box family members. It has been shown that Tbx2 is expressed in the inflow tract, atrioventricular canal, inner curvature and outflow tract. It represses the chamber differentiation via atrial natriuretic

factor (ANF) (Christoffels *et al.*, 2004). In fact, Tbx-2 expression domain is complementary to ANF, connexin Cx40, Cx43 and Chisel. Therefore, chamber myocardium development is regulated by the inihibitory activity of Tbx2 and the expression of ANF (Christoffels *et al.*, 2004). Tbx3 is expressed in the developing conduction system, in the sinoatrial node, atrioventricular region, internodal region and atrioventricular region (Hoogaars *et al.*, 2004). Tbx3 represses chamber-specific program gene expression to allow the formation of the conduction system (Hoogaars *et al.*, 2004). Tbx3 is also involved in the specification and formation of the sinoatrial node and regulates the pacemaker gene expression program (Hoogaars *et al.*, 2007). Tbx20 and Tbx5 collaborate with Nkx2.5 and GATA-4 to regulate the chamber specification programm (Hoogaars *et al.*, 2007).

1.3.3.3 Endocardium

Endocardium is the endothelial innermost cell layer in the heart tube and is evolutionary present only in vertebrates (Perez-Pomares *et al.*, 2009) endothelium and therefore endocardium are vertebrate-specific. Endocardium plays an important role in the heart development. In particular, it is involved in the formation of the cardiac valves and septa, the division of the truncus into the aortic and pulmonary trunks, the development of Purkinje fibers and the formation of trabecular myocardium (Harris and Black, 2010). A group of cells from specific region of the endocardium undergoes a endocardial-mesenchymal transformation (EMT) to form the endocardial cushions which grow in multiple locations during of the heart to form the valvular structure and the membranous septan (Harris and Black, 2010; Person *et al.*, 2005). The *Xenopus* Smad3 is of interest as within the heart it is only expressed in the endocardium from stage 27 to 33 (Howell *et al.*, 2001).

1.3.4 Cardiac differentiation factors

The heart differentiation is characterised by the regional expression of genes such as MLC2, cTnI, MLC1v, Irx4 and ANF which are specifically expressed in the atrial and ventricles chambers. MLC2 expression in *Xenopus* starts at late tail bud stages (stage 26) when the cardiac precursors fuse in the ventral midline and continues to be expressed in the adult heart (Latinkic *et al.*, 2004). Therefore, MLC2 is a pan-myocardial marker. In mammals MLC2 shows a different expression. MLC2a and MLC2v are expressed in the atrial and ventricle chambers respectively (Latinkic *et al.*, 2004; Small and Krieg, 2004). It has been shown in *Xenopus* that MLC2 is homolog of MLC2a in mammals and no equivalent of MLC2v has been found in *Xenopus* (Latinkic *et al.*, 2004).

Cardiac Troponin I (cTnI) is a heart specific marker. Its expression is restricted to the heart in all developmental stages. It is first expressed in the heart at tailbub stage (stage 26) and continues to be expressed after late developmental stages (stage 40) (Drysdale *et al.*, 1994).

MLC1v is a regionalised myocardial marker which is expressed at tailbud stage in the somitic mesoderm. During heart tube formation is expressed in the anterior half of the prospective myocardium, in the bilateral lymph heart and in the precursors of the interhyoid muscles of the jaw (Smith *et al.*, 2005). After heart looping (stage 36) MLC1v is expressed in the jaw muscle and in the heart becomes specifically expressed in the ventricle chamber. MLC1v is highly expressed in the proximal outflow tract and in the single ventricle but expression is not found in the atria (Smith *et al.*, 2005).

Iroquois (Irx4) homeodomain transcription factor is detected at stage 20 in a small cluster of cells in the hindbrain and at stage 26 is highly expressed in the hindbrain above the otic vesicle. At stage 28 is expressed also in the retina and only at stage 36 is expressed at low levels in the heart in particular in the lateral sub-region of ventricular myocardium. At late developmental stages (stage 39) it becomes specifically expressed in the ventricle chamber (Garriock *et al.*, 2001; Smith *et al.*, 2005)

Atrial natriuretic factor (ANF) is expressed in the developing myocardium at stage 32 and in both atria and ventricle chambers until stage 45. At stage 47-49, ANF expression becomes specifically restricted in the atria while a decreased expression is observed in the ventricle. At stage 49 ANF is expressed exclusively in the atrium (Small and Krieg, 2000).

1.4 Study of heart development through in vitro and in vivo organogenesis

The heart development is regulated by a core of evolutionary conserved transcription factor in all vertebrate organisms (Olson, 2006). This evolutionary conservation allowed the use of different model organisms to study the multiple aspects that regulate heart development. In our study we wanted to investigate the regulation of different aspects after cardiac specification such as cardiac cell fate diversification and morphogenesis. In order to achieve these goals we used *Xenopus* embryos as a model organism. One of the major advantages of using *Xenopus* to study the heart development is the availability of explant assays such as animal cap assay. The animal cap is pluripotent as under specific induction conditions they can generate numerous cell types. For example, treatment of the animal cap explants with different concentration of activin induces the formation of different mesodermal tissues in a dose dependent manner (Ariizumi *et al.*, 1991; Asashima *et al.*, 1990; Green *et al.*, 1990). Recent evidence suggests that this simple assay can also be used for *in vitro* organogenesis studies. Latinkic and colleagues, demonstrated that injection of GATA-4 mRNA in animal cap explants induces the expression of cardiac differentiation marker MLC2 and the development of a beating tissue (Latinkic *et al.*, 2003). In this work, using GATA-4 injection in animal cap explants we have proved the possibility to investigate the cardiac patterning and cell diversification establishment in cultured ectodermal explants. However, animal cap assay does not provide a reproducible method to investigate the mechanism of cardiac morphogenesis, as their capacity to beat in culture is poor. In addition, the beating explants only show a very limited degree of morphogenesis. Thus, in order to investigate the mechanisms that regulate heart morphogenesis a reliable assay was needed in which it was possible to obtain a heart-like structure with high degree of morphogenesis and beating capacity. For this reason we

combined and modified the animal cap assay and the secondary heart formation assay (Ariizumi *et al.*, 2003) where injected explants were transplanted into host embryo. Using *in vivo* heart formation assay, we have proved the possibility to investigate different aspects involved during heart morphogenesis through the generation of a secondary beating structure with features resembling the heart proper.

1.4.1 Thesis aims

We aimed to study the mechanism involved in the cardiac cell diversification establishment using animal cap explants previously injected with GATA-4. We wanted to verify if, under the inducing activity of GATA-4, we could find in the same explants the expression of ventricular versus atria markers and if those genes showed a patterning of expression. Moreover, we also aimed to study the different aspects concerning the heart morphogenesis. In order to achieve that, we combined an *in vivo* and an *in vitro* assay, in which GATA-4 injected dissociated and reaggregated AC explants were transplanted into a host embryo at specific developmental stages. The aim to use this second assay, heart formation assay, was to combine our study on cardiac cell diversification establishment with the mechanism involved during the heart morphogenesis. We also tried to study different aspects of the heart morphogenesis using *Xenopus* whole embryos. We aimed to use specific inhibitors of pathway of signals in whole embryo at developmental stages corresponding to the end of the cardiac specification. Using treated whole embryos we aimed to verify which pathway of signals are involved during the different steps of the heart morphogenesis.

2. *Material and Methods*

2.0 Material and Methods

Chemicals, salts, buffer and equipments were obtained from Bio-Rad (California, USA), Roche Diagnostic (Mannheim, Germany), Fisher Scientific (Loughborough, UK), Invitrogen (Paisley, UK), Ambion (Cambridgeshire, UK), Promega (Madison, USA), Sigma-Aldrich (Dorset, UK), GE Healthcare (Buckinghamshire, UK) and New England BioLabs (Ipswich, UK).

2.1 Xenopus laevis embryos manipulation

2.1.1 Obtaining Xenopus laevis embryos

Xenopus embryos were obtained as previously described (Sive *et al.*, 2000) either by natural mating or manual eggs collection. Adult frogs were injected with Human Chorionic Gonadrotopine hormone (HcG, Sigma-Aldrigh) via the dorsal lymph sac. The concentration was dependent upon the frog size, generally between 700-800U for female and 200U for male. Male and female were allowed to mate naturally in a tank over night at 19°C after hormone injection, and the embryos were collected manually with a plastic pipette. Before manipulations embryos were treated with 2% cysteine (pH 7.9-8.0, Sigma-Aldrigh) for 5-10 minutes (min) to completely remove the protective jelly. After de-jellying, embryos were washed in 10% Normal Amphibian Media (NAM; 110mM NaCl, 2mM KCl, 1mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.1mM EDTA, 1mM $NaHCO_3$) and stored at 14°C until required.

For *in-vitro* fertilisation, female frogs were injected with HcG and eggs were obtained by manual release into a Petri dish. The male frog was sacrificed and the testes was surgically isolated from the abdomen using forceps and kept in Leiboviz's L15 media (Sigma-Aldrigh) at 4°C for up to 5 days. Small explants of testes was sliced and eggs coated, and left for 5 min for fertilisation to take place which is indicated by contraction of pigmentation. Embryos were left for 20 min in 10% NAM to rotate and after fertilisation were de-jellied in cysteine (2%, pH 7.9-8.0, Sigma-Aldrigh) as previously described.

2.1.2 Xenopus strain

Experiments were carried out using two *Xenopus* transgenic lines carrying a cardiac actin-GFP reporter (Latinkic *et al.*, 2002) or an MLC1v-GFP reporter (Smith *et al.*, 2005). Green Fluorescent Protein (GFP) presence was detected using a Leica MZ16F Fluorescence Stereomicroscope (Milton Keynes, UK) with a GFP filter (GFP2: excitation 480/40 nm, emission 510 nm). GFP protein emits fluorescence at wavelength of 510nm.

2.1.3 Staging of embryos

Embryos were staged according to the Normal table of *Xenopus laevis* stage development (Nieuwkoop and Faber, 1994). Developmental stages of cultivated explants were assessed according to stages of development reached by siblings control embryos.

2.1.4 Injection of Xenopus laevis embryos with mRNA

Embryos were kept in 10% NAM at 16°C until the required embryonic stages. Embryo injections were performed in Petri dishes containing 75% NAM, 3% Ficoll PM 400 (Sigma-Aldrigh). Needles were pulled from capillary (Harvard Apparatus) using a Kopf 720 Neddle Puller (Kopf Instruments, CA, USA). Samples were filled into the needle using IM 300 microinjector machine (NARISHIGE). After needle calibration for each embryo was injected 10nl of mRNA solution using 10mm graticule (Tonbridge Kent, England). Together with the mRNA, a final concentration of 10%, rodamine-dextran (20mg/ml) and dextran-biotin (25mg/ml) (Molecular probes) was co-injected as lineage tracer (table 2.2).

Embryos were injected at 1-2 cell-stage and kept in 75% NAM, 3% Ficoll PM 400 until stage 9 in order to be processed for animal cap isolation. Embryos injected for phenotypic analysis were transferred to 10% NAM four hours after injection in order to avoid defective gastrulation due to high the salt content of 75% NAM.

2.1.5 Animal cap isolation

Animal caps were cut as described by Sive and colleagues at stage 9 in agarose-coated dishes (1% agarose in dH_2O) containing 75% NAM (Sive *et al.*, 2000).

Briefly, embryos at stage 9 show a distinct morphology composed of two defined poles, the animal pole, in which the mRNA was previously injected, and the vegetative pole at the opposite extremity. The animal pole is composed of a group of pigmented undifferentiated cells which cover a cavity called blastocoel. The vegetal pole is the region containing the yolk which provides the nutrients for the growing embryo. Cutting animal caps first involved

removing the chorion membrane which surrounds the whole embryo. Animal pole explants were excised from the embryo and incubated in 75% NAM with gentamicin (25mg/ml, Sigma-Aldrigh) and also a mix of streptomycin-penicillin (50X, Sigma Aldrich) at 19-21°C. Due the use of an inducible form of GATA-4 mRNA dexamethasone hormone (Dex, Sigma-Aldrich) was added over night to the explants (2μM) to activate GATA-4-GR (Latinkić *et al.*, 2003). Control embryos were kept in 10% NAM and gentamicin (25mg/ml, Sigma-Aldrich) at the same temperature as animal caps until the required developmental stage.

2.1.6 Dissociation and reaggregation of animal cap explants

Animal cap explants were cut at stage 9 in agarose-coated dishes (1% agarose in dH_2O) containing 95% Ca^{2+}/Mg^{2+}- free saline solution (CMFM; 88mM NaCl, 1mM KCl, 2.4mM $NaHO_3$, 7.5mM Tris-HCl pH 7.6) and 75%NAM. Twenty five explants were transferred to a 1.5ml tube and dissociated in CMFM, 0.1% Bovine Serum Albovine (BSA 99%, Sigma-Aldrige). The dissociated cells were centrifuged at 1000 rpm for 1 minute and CMFM, 0.1% BSA was replaced with CMFM, 0.5mM $CaCl_2$ (fig. 2.1 A) Dex was added to the dissociated cells injected with GATA-4 mRNA. The cells were left for 3 hours at 19-21°C. The reaggregate cells (reaggregates) were transferred to agarose-coated dishes containing 75%NAM and gentamicine (25mg/ml, Sigma-Aldrich) and left over-night at 19-21°C (fig. 2.1 A) (Kuroda *et al.*, 2005; Sive *et al.*, 2000).

2.1.7 Transplants of reaggregates in Xenopus host embryos

Transplants were originally carried out according to Ariizumi and colleagues (Ariizumi *et al.*, 2003). Reaggregates at stage 20 were transplanted into host embryos at the same stage. Embryos were transferred to agarose-coated dishes (1% agarose in dH_2O) containing 75%NAM with gentamicine (25mg/ml, Sigma-Aldrich). The chorion was removed from the embryos using a pair of forceps. The reaggregates were transferred to the same agarose dish and using a syringe needle and a forceps were cut to 0.03 mm in size using a 10mm graticule (Tonbridge Kent, England) placed under the agarose dish as a guide. In the ventral region of the host was created a wound where the reaggregate was transplanted. Transplanted host embryos remained in the agarose dish at least for one hour in order to allow the wound to repair and minimising the possibility of rejection (fig 2.1 B).

2.1.8 Morphogenic analysis of reaggregates by confocal microscope

Confocal analysis was carried out on reaggregates transplanted into host embryos using a fluorescent microscope (Leica DM6000B). All images were analysed with the same magnification using objective lense HC PL FLUOTAR 40X. Leica Confocal Software 2.0 was used in order to process the images during secondary heart (SH) scanning.

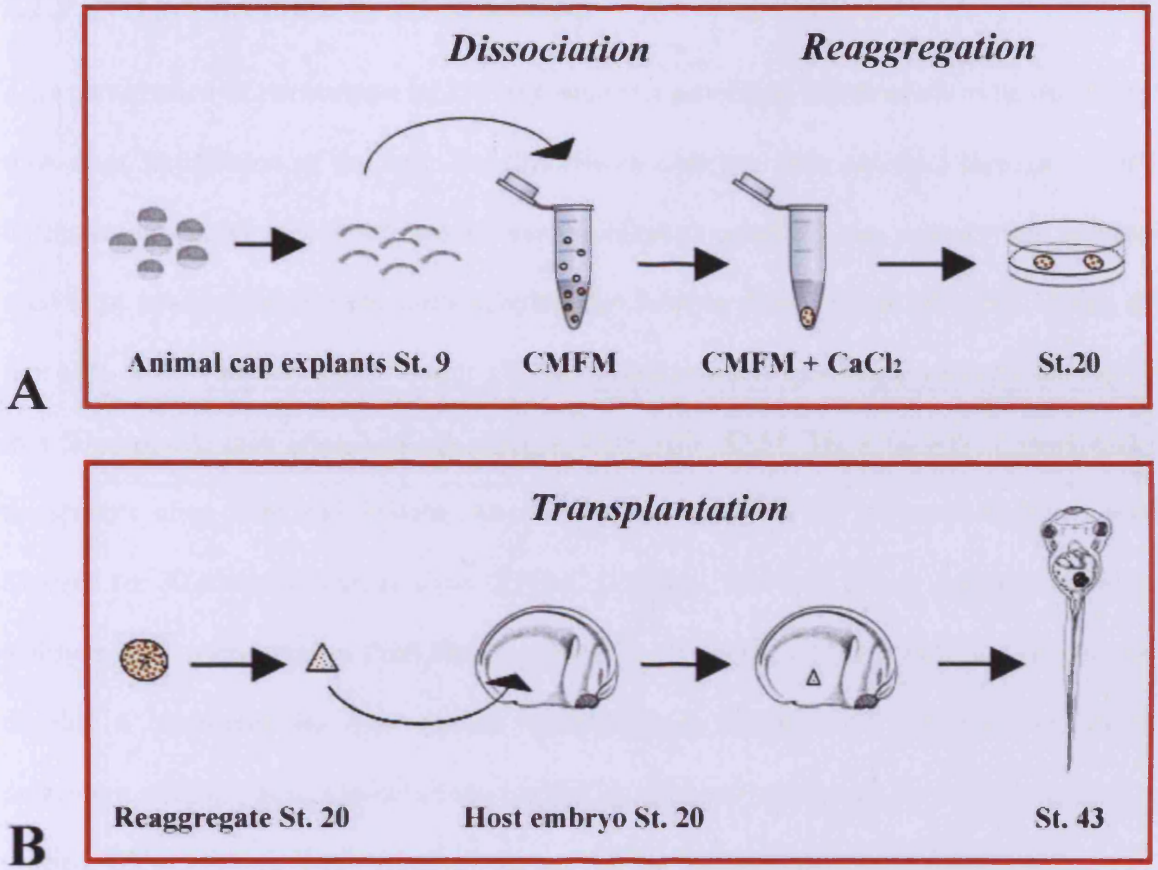

Figure 2.1 Dissociation and reaggregation of explants transplanted in host embryos. (A) Animal cap explants previously injected were excised from the animal pole and dissociated in CMFM and after reaggregation were cultivated until stage 20. (B) Transplantations were performed on the ventral side of the host embryos at stage 20 with reaggregates at the same stage. Transplants were analysed at stage 43.

2.1.9 Axis perturbation by UV-treatment

Axis perturbation of the embryo by UV exposure is a procedure which needs to be carried out soon after fertilisation of the egg. For this reason embryos were obtained through *in vitro* fertilisation as previously described. In order to obtain a complete axis perturbation, embryos need to be irradiated before the cortical cytoplasm rotation (CCR) is completed i.e. during the first 60% of the first cell cycle (Sive *et al.*, 2000). Immediately after, embryos were transferred to a 50ml plastic tube (Anachem) containing 45ml 10% NAM. The tube was covered with a transparent cling film and inverted upside down over a UVGL-25 lamp. Embryos were exposed for 30 seconds to short wave (254nm) UV-light. Five min after irradiation treatment, embryos were transferred to Petri dishes containing 10%NAM. Good quality ventral embryos develop a blastopore lip that appears simultaneously around their entire circumference, coincident with the development of the ventral lip in control embryos. An accurate method to scoring UV-treated embryos involves the use of the dorsoanterior index (DAI) (Sive *et al.*, 2000). DAI is given as a whole number. Irradiated embryos with DAI inferior to 2 were chosen for further experiments (Sive *et al.*, 2000). UV-treated embryos were used as host at stage 20 for the transplantation of GATA-4 injected reaggregates at the same stage.

2.1.10 Heart field precursor excision and dissociation

Embryos at stage 21 were transferred to an agarose coated dish (1% agarose in dH_2O) with 75% NAM and the chorion was removed using of a pair of sharp forceps. Heart Fields (HF) were dissected from the embryo using a syringe needle and a sharp forceps (fig. 2.2 A). The HFs were transferred to an agarose coated dish (1% agarose in dH_2O) with 75% NAM and analysed at St. 40.

HFs dissociation was carried out in an agarose coated dish (1% agarose in dH_2O) with 95% Trypsin (Sigma-Aldrigh) and 5% of 75% NAM (fig. 2.2 B). HFs were left 10-15 min in order to allow the cell to dissociate completely and transferred in 1.5ml tube. The dissociated cells were centrifuged at 1000rpm for 30 seconds and trypsin was replaced with 75% NAM and 0.5mM $CaCl_2$. The dissociated cells were centrifuged at 1000rpm for 30 seconds and left at least three hours to reaggregates before transferring to an agarose coated dish (1% agarose in dH_2O) containing 75% NAM.

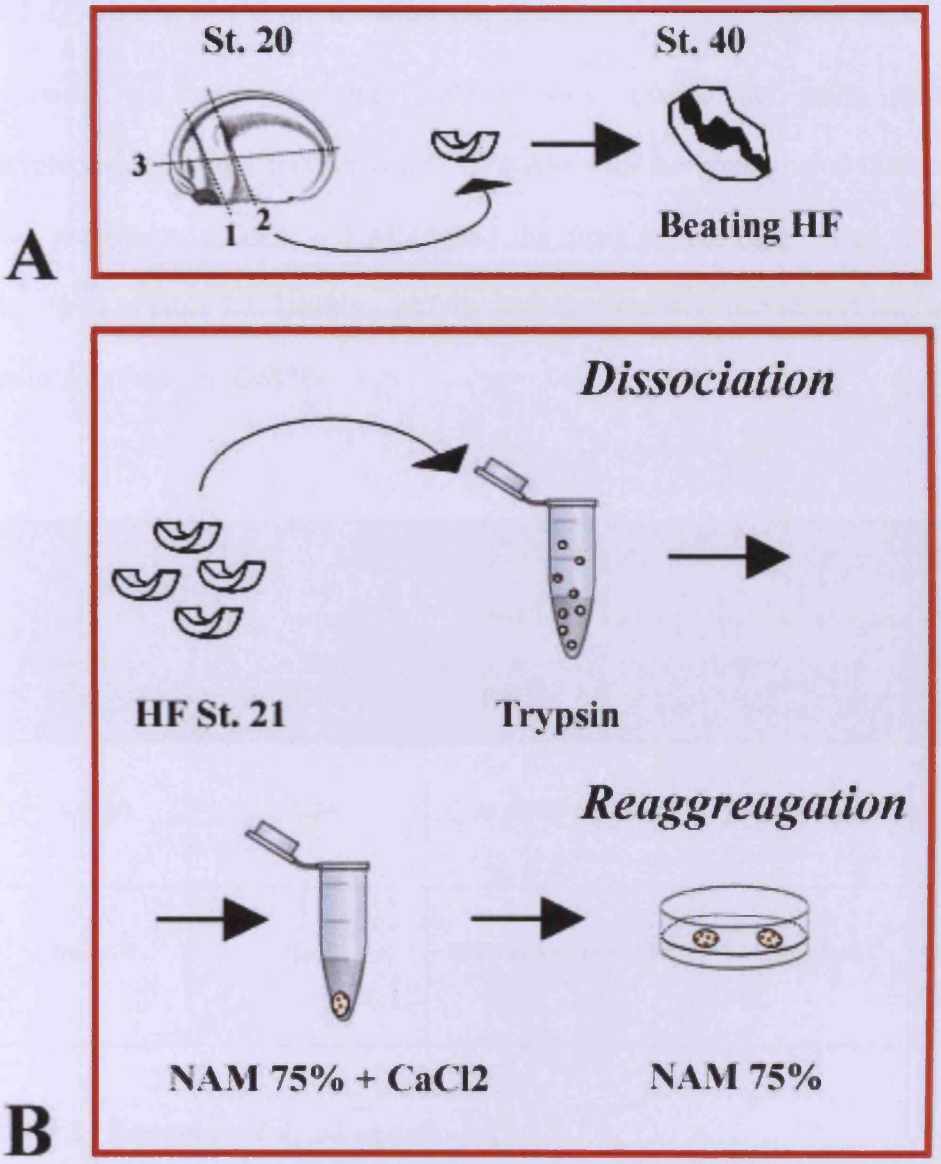

Figure 2.2 HF dissection, dissociation and reaggregation. (A) The HF was excised from embryos with two transverse sections (1 and 2) and a further longitudinal section (3) and cultivated until beating activity was detected. (B) The HF at stage 21 were dissociated using trypsin and reaggregated in 75% NAM and $CaCl_2$ and cultivated in agarose coated dishes (1% agarose in dH_2O) with 75% NAM.

2.1.11 Media inhibitor treatment

Inhibition of specified signal pathways was carried out using embryos at specific developmental stages incubated in 75% NAM with the appropriated inhibitor. Each inhibitor was previously diluted in DMSO and the final concentration used in each treatment is described in table 2.1. Inhibitor activity was confirmed by phenotypic analysis compared with control embryos in DMSO.

Inhibitor	Concentration	Mode of Action	Manufacturer	References
A-83-01	75µM	ALK4/5/7 inhibitor	Sigma-Aldrigh	(Tojo *et al.*, 2005)
SU-5402	50µM	RTK inhibitor of FGFR1-4	Calbiochem	(Mohammadi *et al.*, 1997)

Table 2.1 Inhibitors of signaling pathway.

2.2 RNA synthesis

2.2.1 Linearisation of DNA template

Constructs of different DNAs were linearised using the appropriate restriction enzyme as described in Table 2.2. DNA constructs (5µl) were incubated with the restriction enzyme and enzyme specific buffer at the appropriate temperature according to the manufacture specifications. Construct linearisation was confirmed using agarose gel electrophoresis made with TBE buffer (45mM Tris Base, 45mM Boric Acid, 1mM EDTA ph 8.0, 0.1% Acetic Acid) and ethidium bromide incorporation (6µl for 100ml of agarose, Fisher) run at 75V for 30 min. DNA linearisation was visualised putting agarose gel under UV light (Gel Doc-It Immage System, Biorad). DNA molecular weight was verified using a 1Kb marker (Invitrogen). The enzyme reaction was inactivated by incubation of the mix at 65°C for 20 min.

Plasmid	Construct	Linearisation	RNA polymerase	mRNA injection	References
GATA-4-GR	pCS2	SalI	Sp6	1ng/10nl	(Afouda *et al.*, 2005)
Dkk-1	pCS2	NotI	Sp6	1ng/10nl	(Glinka *et al.*, 1998)
tBr	pSP64T	EcoRI	Sp6	1ng/10nl	(Graff *et al.*, 1994)
XWNT8	pCSKA	BamHI	Sp6	800pg/10nl	(Christian and Moon, 1993)

Table 2.2 DNA used for RNA synthesis. Plasmid construct with their restriction sites and polymerase used to generate mRNA are described.

2.2.2 mRNA synthesis

Capped RNA was synthetised from linearised templates and the Sp6 promoter was used to drive the transcription reaction (table 2.2). The reaction was performed using the 7mG(ppp)G RNA Cap Structure Analogue (NEB). Briefly, DNA (2µl) was incubated with transcription buffer (400mM Tris pH 7.5, 60mM $MgCl_2$, 20mM spermidine HCl, 50mM NaCl, 2µl), 12.5 mM DTT, 1mM dNTPs, RNAse Inhibitor (RNasin, Promega) and 40 units of Sp6 (Ambion) and incubated for 2 hours at 37 °C. This was followed by addition of RQ1 RNAse-free DNase (Ambion) and incubation for 15 min at 37 °C. Transcribed RNA was purified using ProbeQuant™ G-50 Micro Columns (GE Healthcare) and run on agarose gel (1%) stained with ethidium bromide to check its integrity. The concentration of the RNA was determined using a spectrophotometer (Bio-Rad Smart Spec 3000).

2.2.3 Dexamethasone inducible construct activity

Glucocorticoid receptor (GR) fusions provide stable constructs which can be used to create sense RNA which can be injected into *Xenopus* embryos. Fusion to the receptor renders the protein inactive due to its interaction with Heat Shock Protein 90 (HSP 90) complex. Fused proteins accumulate in the nucleus until its ligand dexamethasone (Dex) is added. Dex causes a conformational change that release HSP90 from the protein which is thereby activated (Mattioni *et al.*, 1994).

2.3 RT-PCR analysis

2.3.1 mRNA extraction

RNA extraction was carried out using acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Twenty five animal cap explants, 3 reaggregates, 5 HF explants or 5 control embryos were used per sample. Briefly, denaturing solution (Solution D; 4M guanidinium thiocynate, 25mM sodium citrate, pH7.0) containing 0.1M β-Mercaptoethanol (98%; Sigma-Aldrigh) was added to each sample. Sequentially, 2M sodium acetate (pH 4, 50µl), water saturated phenol (500µl, Fisher Scientific) and chloroform (100µl, Fisher Scientific) were added to the samples homogenate and mixed, followed by incubation on ice for 15min. Samples were centrifuge at 14,000 rpm for 20 min at 4°C. Ice-cold isopropanol (500µl, Fisher Scientific) was added to the subsequent solution and incubated for 1 hour at -70 ° C. Following centrifugation at 14,000 rpm for 20 min at 4° C, the solution was removed and cold 70% ethanol (500µl, Fisher Scientific) added and centrifuged as before. Pellets were air-dried and resuspended in ddH_2O (0.5µl for each animal cap) and stored at -20°.

2.3.2 Reverse transcription

Reverse transcription polymearase chain reaction was carried out using M-MLV Reverse transcriptase kit (Invitrogen) according to the manufactures specifications. Briefly, 2µg of RNA, 1.25µl dNTPs (10mM, Promega) and 1µl random primers (0.5µg/µl, Invitrogen) were added to a volume of 15 µl. To denature the RNA, samples were incubated at 65°C for 5 min

and then quickly cooled on ice. The following mix was then added to each sample and incubated for 2 min at 37°C.

5µl	5X First Strand Buffer (250mM Tris-HCl pH 8.3, 375 mM KCl, 15mM $MgCl_2$)
2.5µl	DTT (100mM)
1.25µl	RiboLock RNase Inhibitor (40U/µl, Fermentas)

1.25µl M-MLV RT (200 units, Invitrogen) was added to each sample and incubated for 10 min at 25°C followed by an incubation of 50 min at 37°C. The reaction was heat inactivated for 15 min at 70°C and the cDNA stored at -20°C.

2.3.3 RT-PCR

The expression of the genes of interest in treated animal cap explants, reaggregates or whole embryos was analysed using RT-PCR. RT-PCR was performed using the primers described in table 2.3 at concentration of 0.1µM. Primers (0.1µM) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). PCR conditions and primers sequence are described in table 2.3. Cycle number and reaction condition were scrutinised such that production amplification was determined to be in the linear range of specific PCR product. Reactions were set up in duplicate for each cap sample using the following mix:

5µl	5X GoTaq Flexi Buffer (Promega)
0.5µl	dNTPs (10mM)
2µl	$MgCl_2$ (2mM, Promega)
1µl	Forward primer (2.5mM, Invitrogen)
1µl	Reverse primers (2.5mM, Invitrogen)
0.25µl	GoTaq DNA Polymerase (5U/µl, Promega)
to 24µl	ddH_2O

PCR was carried out using MJMini PCR Machine (Bio-Rad) with the following cycling conditions:

95°C for 3 min

95°C for 30 seconds
Tm for 30 seconds
72°C for 30 seconds
} x cycles

72°C for 10 min

Specific cycle numbers were performed for each gene analysed as described in Table 2.3. Following PCR, products were analysed using agarose elecrophoresis gel (2%) with ethidium bromide staining visualised under UV. Samples were normalised relative to Ornithine Decarboxylase (ODC) levels as a loding control.

Primers	Sequence	Accession number	Tm (°C)	Product Size (bp)	Cycle Number
IRX4 (+) IRX4(-)	tgcagctttgggtgtctatg atggccagcatgatcttctc	NM_001096735.1	58	336	38
MLC1v (+) MLC1v (-)	tgggacagaatccaaccaat tgaatggtgttcctgtgcat	AY289206	58	491	38
MLC2 (+) MLC2(-)	tgtatcgaccaaaaccgtga attggggtcacagcaaacat	AY219706	58	329	32
ODC (+) ODC (-)	gccattgtgaagactctctccatt ttcgggtgattccttgccac	NM_001086698	58	220	28
Tbx18 (+) Tbx18 (-)	tgtttccagccatgagagtg gagagatggctccaaaatgc	EB736397	58	553	39

Table 2.3 Primers used in RT-PCR.

2.3.4 QRT-PCR

Quantitative RT-PCR analysis was performed using cDNA prepared as described previously. Primers (0.1μM) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/), PCR conditions and primers sequence are described in table 2.4. The following mix was used for each PCR reaction with a final volume of 25μl:

5μl	5X GoTaq Flexi Buffer (Promega)
0.5μl	dNTPs (10mM)
2μl	$MgCl_2$ (2mM, Promega)
1μl	Forward primer (2.5mM Invitrogen)
1μl	Reverse primers (2.5mM Invitrogen)
1.25μl	SYBR Green (2X SYBER Green Supermix, BioRad)
0.25μl	GoTaq DNA Polymerase (5U/μl, Promega)
to 24μl	ddH_2O

PCR was carried out using a MiniOpticon PCR Machine (Bio-Rad) with the following cycling conditions:

95°C for 3 min

95°C for 30 seconds
Tm for 30 seconds
72°C for 30 seconds
75°C for 15 seconds
Plate Read
} X cycles

Melting curve from 55°C to 95°C

Data was analysed using MJ Opticon Monitor 2.0 software (BioRad). The fold difference in gene expression levels between samples was calculated with the following formula:

$$\text{Amount of target} = 2^{-\Delta\Delta Ct} \text{ (Livak and Schmittgen, 2001)}$$

Ct correspond to the threshold cycle for target amplification

$\Delta\Delta Ct$ correspond to $Ct_{\text{target gene}} - Ct_{\text{control}}$

Primers	Sequence	Accession number	Tm (°C)	Product Size (bp)	Cycle Number
IRX4 (+) IRX4(-)	acctctggacaccaccaatc cgcaacaggttgaaagtgaa	NM_001096735.1	58	184	45
MLC1v (+) MLC1v (-)	tgctccaacacatttccaag actcttcttgcccatgcagt	AY289206	58	186	45
MLC2 (+) MLC2(-)	tgtatcgaccaaaaccgtga cttctgggtccgttccatta	AY219706	58	189	45
ODC (+) ODC (-)	gccattgtgaagactctctccatt ttcgggtgattccttgccac	NM_001086698	58	220	45

Table 2.4 Primers used in QRT-PCR. Primers used for QRT-PCR required a product size not longer than 250bp. For this reason primers sequence for Irx4, MLC1v and MLC2 were specifically designed for QRT-PCR accordingly.

2.4 Gene expression analysis

2.4.1 Antisense probe preparation

Standard antisense probe synthesis was performed according to the method of Sive (Sive *et al.*, 2000), transcribed from linear DNA. Riboprobes were labelled with digoxygenin- or fluorescin- UTP (Dig or Flu respectively, Roche). DNA templates used were linearised at appropriate promoter site for antisense direction using T7 polymerase (Promega) (table 2.5). Briefly, the reaction mix for each sample (40µl of final volume) contained:

8µl	5X transcription buffer (Promega)
4µl	1M DTT (Promega)
2µl	10X Dig/Flu RNA labelling mix (Roche)
0.5µl	RiboLock RNase inhibitor (40U/µl, Fermentas)
2 µl	RNA polymerase (Tab.2.3)
1 µg	DNA
to 40µl	ddH_2O

The mix was incubated for 2 hour at 37 °C and then 2µl of RQ1 RNAse-free DNase 1U/µl (Promega) was added for 15 min at 37 °C. Probes were purified using ProbeQuant™ G-50 Micro Columns (GE Healthcare) and RNA integrity was checked using agarose gel electrophoresis (1%) with ethidium bromide staining.

Gene	Plasmid	Polymerase	Restriction enzyme	References
MLC2	pGemTeasy	T7	SalI	(Chambers *et al.*, 1994)
MLC1v	pSPORT	T7	SalI	(Smith *et al.*, 2005)
Tbx18	pGemTeasy	T7	SalI	(Jahr *et al.*, 2008)
Cardiac Troponin	Bluescript SK	T7	SalI	(Drysdale *et al.*, 1994)

Table 2.5 Antisense probe used in whole mount in situ hybridisation. Each probe was labelled with either digoxygenin or fluorescein.

2.4.2 Whole mount in situ hybridisation

Animal cap explants and embryos were fixed in MEMFA (0.1M MOPS pH7.4, 2mM EDTA, 1mM $MgSO_4$, 0.1x Formaldehyde) at the required stage (Sive *et al.*, 2000), dehydrated in 100% ethanol (Fisher Scientific) and stored at -20°C until required.

Samples were gradually rehydrated in a TBS ethanol series, washed in TBS-Tween 20 (TTw, 20mM NaCl, 5mM Tris-Cl pH 7.4, 0.1% Tween-20 [Fisher Scientific]) and permeabilised with Proteinase K in TBS (10µg/ml, Roche) for 15 min. After three washes in TTw, samples were fixed in MEMFA for 20 min. Prehybridisation was carried out by incubation in hybridisation buffer (50% Formamide [Fisher Scientific], 5X SSC [SSC, 0.1M NaCl, 15mM Sodium Citrate], 5mM EDTA pH8.0, 10%CHAPS [Fisher Scientific], 1X Denhart's solution [0.02% BSA, 0.02% polyvinylpyrrolidone], 0.02% Ficoll 400, Heparine Sulphate [1mg/ml, Sigma-Aldrigh], Torula RNA Type IX [1mg/ml, Sigma]) at 60-65°C for at least 4 hours.
Samples were rinsed with fresh hybridisation buffer (500µl) containing digoxigenin or fluorescin anti sense probe and incubated at 60-65°C over night.

The samples were then washed once in 25% Formamide, 2X SSC, 0.1% Chaps for 10 min at 60-65°C, followed by two washes in 2XSSC, 0.1% Chaps at 60-65°C for 15 min and several washes in 0.2X SSC, 0.1% Chaps at 60-65°C each one for 30 min. Samples were then washed twice in MAB-T (0.1 M maleic acid [Sigma], 0.15 M NaCl, 0.1 triton [Fisher Scientific]). Blocking was carried out by incubation for at least 1 hour at room temperature in MAB-T containing 2% BMB blocking Reagent (Roche) and 10% Heat Inhactivated Sheep Serum

(HSS, Sigma). Samples were left over night at 4°C in MAB-T, 2% BMB and 10% HSS with either antidigoxigenine antibody (Roche) 1:2500 or antifluorescine antibody (Roche) 1:10000.

Samples were washed several times in MAB-T and incubated for 15 min in Alkaline Phosphatase Buffer (APB; 100mM Tris pH9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20, 2mM Levamisole [Sigma]). Colour detection was achieved using the following substrates:

- *5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Roche).* This substrate stains turquoise when used alone. After AP buffer incubation samples were transferred into AP buffer containing BCIP (3.5µl/ml) and left in dark to allow the staining to develop. BCIP can be used also for colour development in double *in situ* hybridisation. Overlapping colour development between BCIP and the secondary substrate generate a strong blue colouration.

- *BMpurple (Roche).* This substrate develops a strong blue colour after the embryos were previously incubated in AP buffer. BMpurple was used only in double *in situ* hybridisation when there is not overlapping expression between two transcripts.

- *Magenta-Phosphate (Sigma).* This reagent stains magenta. Develop very slowly but it produces low background. It is generally used in double *in situ* hybridisation in combination with BCIP. Overlapping expression generate a violet colour clearly distinguishable from magenta and turquoise. In order to obtain a stronger stain it is generally used in combination with Tetrazolium Red (TTZ, Sigma). Stock of Magenta-

Phosphate (25mg/ml) and TTZ are made in Dimethylformamide and used at 9µl/ml in AP buffer.

Once colour development was complete, samples were dehydratated and then rehydratated in an ethanol series in order to remove unbound stain and stop the colour reaction. After washing in MAB-T for 10 min, samples were stored in TBS at 4°C or bleached with 1%H_2O_2, 5% Formammide, 0.5XSSC. Benzyl alcohol;Benzyl benzoate (BB/BA) was used to clear the embryos and animal cap explants.

Double *in situ* hybridisation was carried out for the analysis of two different RNA transcripts. During hybridisation fluorescine and digoxigenine probes were added together. After the development of the first transcript samples were fixed in MEMFA for 20 minute and gradual hydration-dehydration ethanol series was carried out. Samples were washed in MAB-T and blocked for 1h at RT in MAB-T, 2% BMB, 10% HSS. The secondary antibody was subsequently added over night at 4°C and developed as described above.

2.4.3 Immunohystochemistry

Transplant embryos were analysed for the presence of cardiac tissue in reaggregates using mouse anti-chicken tropomyosin monoclonal antibody (CH-1) (Kolker *et al.*, 2000). Transplants were fixed in Dent's fixative (80% Methanol [Fisher Scientific], 20% dimethyl sulfoxide [DMSO]) for 2 hours at RT and bleached using 5% hydrogen peroxidase (in Dent's

fixative) for 4-5 hours at RT. Transplanted embryos were rehydrated with PBS (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, 1%BSA) in a methanol-PBS series with 1% DMSO. Transplants were incubated 30-45 min in 50mM acetate buffer containing bovine testicular hyaluronidase (1mg/ml, Sigma-Aldrigh) and rinsed in PBS-T (PBS, 0.1% Triton-X) three times. Transplants were incubated in PBS-T containing 2% BMB blocking Reagent (Roche) and 5% Heat Inhactivated Sheep Serum (HSS, Sigma) for 4 hours at RT. The primary antibody CH-1 was diluted 1:50 in PBS-T, 2% BMB, 5% HSS and left over night at 4°C. The samples were then washed five times in PBS-T and incubated over night at 4°C in PBS-T, 2% BMB, 5% HSS containing 1:500 dilution of Goat α-mouse Alexa Fluo 488 (Invitrogen) (Kolker *et al.*, 2000). The samples were then washed in PBS-T and fluorescence detection was carried out using a Leica M216 fluorescent microscope (Milton Keynes, UK) with a GFP filter.

For confocal analysis samples were dehydrated in methanol (MEOH), and cleared with benzyl alcohol/benzyl benzoate (BABB). Following this treatment, the tissues were transparent. Samples were transferred to slides coated with 1% agarose in which wells were cut and filled with BABB solution. The embryos were covered with a cover slip in order to prevent evaporation.

3. *Heart cell fate diversification in an animal cap assay*

3.0 Heart cell fate diversification in an animal cap assay

3.1 Introduction

Vertebrate heart development involves a series of morphogenetic steps in which a relatively simple layer of cardiac precursors generates a complex structure composed of atrial and ventricular chambers. This complexity presents a challenge for *in vivo* study of heart development, an essential organ, as very often experimental manipulations cause early lethality. As one alternative, in order to understand how cardiac cell diversification of ventricular versus atrial cells is established during embryogenesis, we used a simple animal cap (AC) assay. AC explants excised from the animal pole of the embryo and cultivated in a simple saline solution express epidermal genes. However, under specific induction with certain molecules they can be induced to differentiate into specific cell types. Latinkic and colleagues showed that injection of GATA-4 is sufficient to induce cardiac differentiation. In these explants the pan-myocardial marker MLC2 is expressed and when cultivated longer also show beating activity (Latinkic *et al.*, 2003). AC explants therefore, provide a simple *in vitro* to study the mechanisms of heart development.

3.1.1 Chapter Aim

Using the cardiogenesis assay, we aimed to:

1. Investigate the establishment of regionally restricted cardiac fates in intact animal pole explants.
2. Investigate the mechanisms that lead to the establishment of regionally restricted cardiac genes.

3.2 Results

3.2.1 Gata-4 injection induces regionally restricted cardiac fates in animal cap explants

It has been previously established that isolated parts (explants) of *Xenopus* embryos are able to grow and differentiate when cultivated in a simple saline solution. It has also been shown that the explants injected with GATA-4 express pan-myocardial markers such as MLC2 but no muscle differentiation markers (Latinkic *et al.*, 2003).

Animal cap (AC) explants cultivated until stage 33 showed the expression of MLC2 when analysed by whole mount *in situ* hybridisation (WMISH) and RT-PCR analysis (fig. 3.1 A). Further analysis has been carried out on explants injected with GATA-4 in order to verify the existence of ventricular markers MLC1v and Irx4. During embryonic development, MLC1v and Irx4 become ventricular specific markers after stage 37 and therefore the explants were cultivated until stage 39. *In situ* hybridisation analysis (fig. 3.1 G-H) showed that 75% of the

explants express MLC1v indicating that ventricular cell fate has been induced. This was confirmed by RT-PCR analysis (fig. 3.1 A) which also showed the expression of Irx-4.

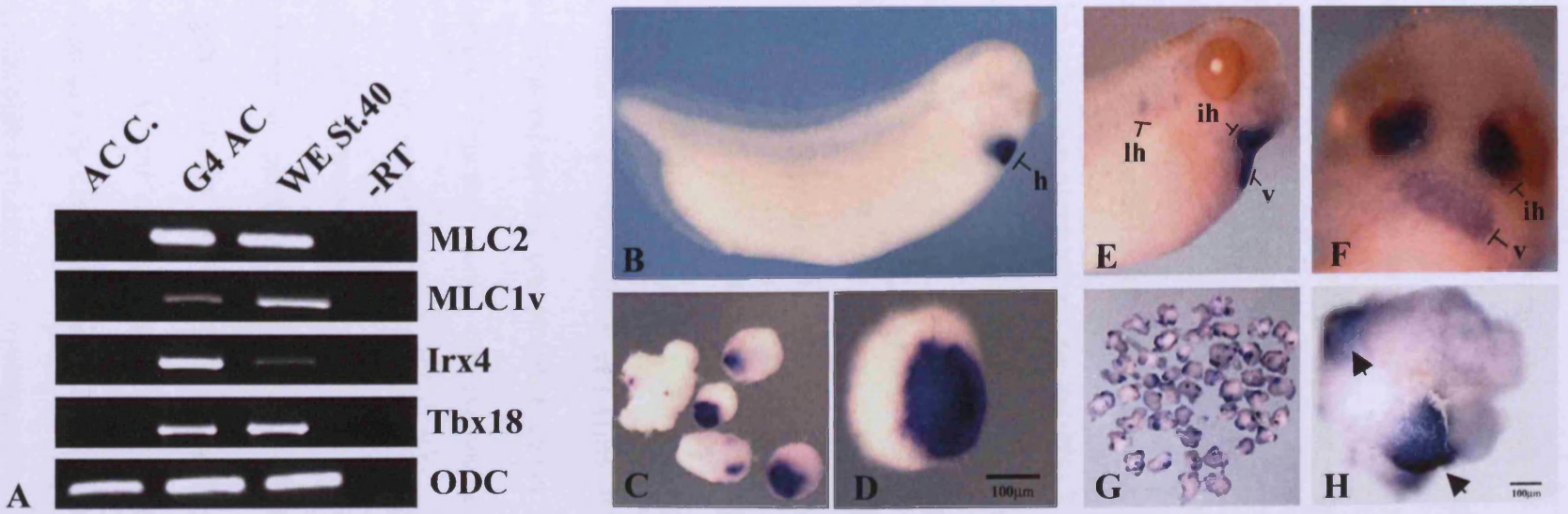

Figure 3.1 GATA-4 injected animal caps express specific ventricular markers (A) GATA-4 injected animal caps cultivated until stage 40 showed the expression of MLC2, MLC1v, Irx4 and Tbx18 when analysed using RT-PCR at stage 40. (B) Control embryo at stage 33 analysed by WMISH for the expression of MLC2 (C-D). 50% of AC explants expressed MLC2 (n=25) when analysed by WMISH. (E-F) Control embryo at stage 40 analysed in WMISH for the expression of MLC1v. (G-H) 75% of AC explants cultivated until stage 40 express MLC1v (n=50) when analysed by WMISH (arrows). ih; interhyoid muscles of the jaw; lh; lymph heart, v; ventricle, AC; animal caps control, G4; GATA-4, WE; whole embryos.

GATA-4 AC explants were also analysed by double WMISH for the expression of pan-myocardial markers and a ventricular marker (fig. 3.2). The lacking of suitable atrial markers in X*enopus* directed us to use the ventricular and panmyocardial markers together as an indirect method to observe also the establishment of atrial cell fate. Double WMISH performed in whole embryos showed overlapping expression between the panmyocardial and ventricular markers which corresponds to the ventricular region, while the region of MLC2 expression only correspond to the atria (fig. 3.2 A1 and B1). 36% of AC explants analysed for the expression of panmyocardial marker cTnI and ventricular marker MLC1v showed a region of overlapping expression between these markers and a region in which cTnI was expressed alone (fig. 3.2 A2-A4). 13% of GATA-4 AC explants express only cTnI. Similarly, 41% of GATA-4 AC analysed for the expression of MLC2 and MLC1v showed a region of overlapping expression between these markers and regions where MLC2 was expressed alone (fig. 3.2 B2-B4). 30% of GATA-4 AC explants expressed MLC2 only. Additional investigation has been carried out in GATA-4 injected explants in order to check for the presence of non-myocardial cell types. RT-PCR analysis demonstrated that GATA-4 AC explants express the proepicardial marker Tbx18 (fig. 3.1A). These results demonstrate that GATA-4 injected animal cap explants express myocardial and non-myocardial markers. Moreover, in the same explants pan-myocardial markers and ventricular marker showed patterned expression when analysed by double WMISH. The overlapping expression found between the panmyocardial markers and the ventricular marker corresponds to the ventricle region while the expression of the panmyocardial markers alone corresponds to the atrial region. Animal cap explants therefore demonstrate a reproducible model that allows us to investigate the establishment of cardiac ventricular versus atrial cell fate diversification during cardiac differentiation and organogenesis.

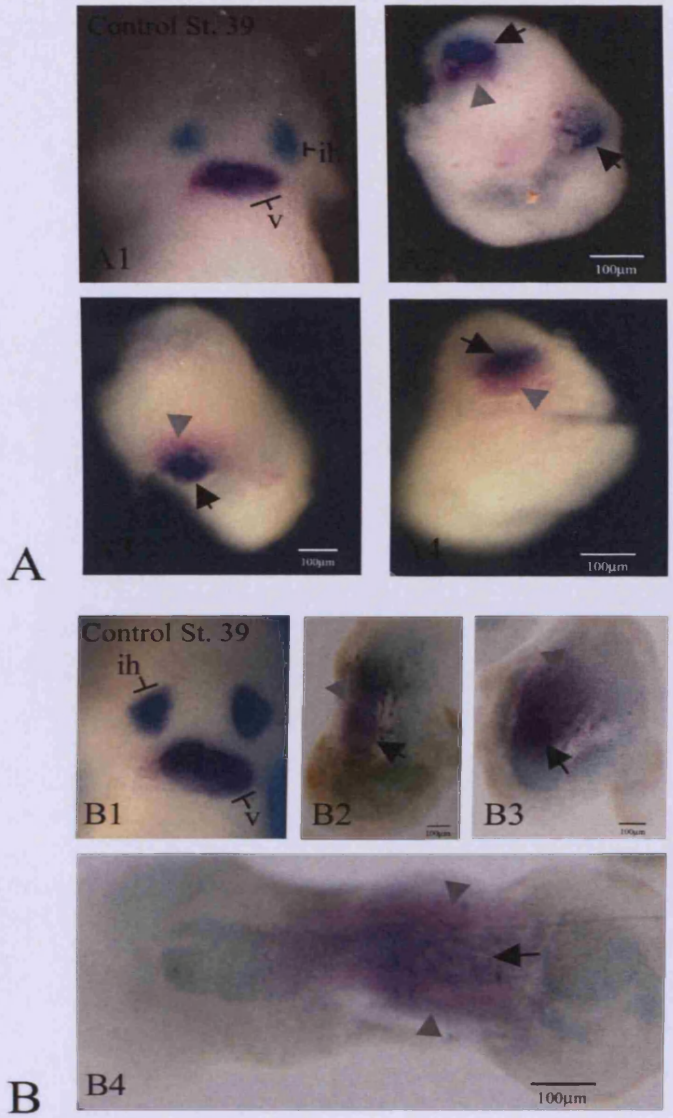

Figure 3.2 Regional gene expression in GATA-4 AC. (A1) Control embryo at stage 39 analysed by WMISH for the expression of cTnI and MLC1v. (A2-A4) 36% of GATA-4 AC (n=55) showed region of overlapping expression between cTnI and MLC1v (arrow) and region of cTnI expression alone (head arrow). (B1) Control embryo at stage 39 analysed by WMISH for the expression of MLC2 and MLC1v. (B2-B4) 41% of GATA-4 AC (n=41) treated by BB/BA showed region of overlapping expression between MLC2 and MLC1v (arrow) and region of MLC2 expression alone (head arrow). ih; interhyoid muscles of the jaw, v; ventricle.

3.2.2 Dkk-1 enhances cardiogenesis but has only a small specific effect on MLC1v

Latinkic and colleagues showed that cardiogenic activity of GATA-4 in animal caps explants can be enhanced by co-injection with Dkk-1 (Latinkic *et al.*, 2003). Dkk-1 is a Wnt/β-catenin antagonist (Semenov *et al.*, 2001) which acts as a head inducer via the anteriosation of the body during embryonic development (Glinka *et al.*, 1998). AC explants were co-injected with Dkk-1 and GATA-4 and analysed using WMISH and RT-PCR to verify if cardiac cell diversification has occurred. Co-injected animal cap explants analysed by WMISH (fig. 3.3 D-E) and RT-PCR (fig. 3.3 D-E) showed the expression of pan-myocardial marker MLC2. qRT-PCR analysis showed that, in co-injected explants, MLC2 expression was enhanced when compared with the expression in GATA-4 injected explants (fig. 3.3 B). Furthermore animal cap explants cultivated until stage 40 showed the expression of ventricular specific markers when analysed by RT-PCR (fig 3.3 A). MLC1v expression was also confirmed using WMISH in which 50% of the explants analysed showed the expression of this gene (fig. 3.3 G-H). qRT-PCR analysis demonstrated also an increase of MLC1v expression in co-injected AC explants compared with GATA-4 injected AC (fig. 3.3 B). Thus, in co-injected explants there is an overall increase in cardiac tissue (MLC2) caused by Dkk-1 that most likely accounts for the increase of MLC1v. GATA-4 and Dkk-1 co-injected explants were also analysed for the expression of Tbx18 in order to verify the presence of non-myocardial cell types. Co-injected explants analysed by RT-PCR do not show the expression of Tbx18 (fig. 3.3 A).

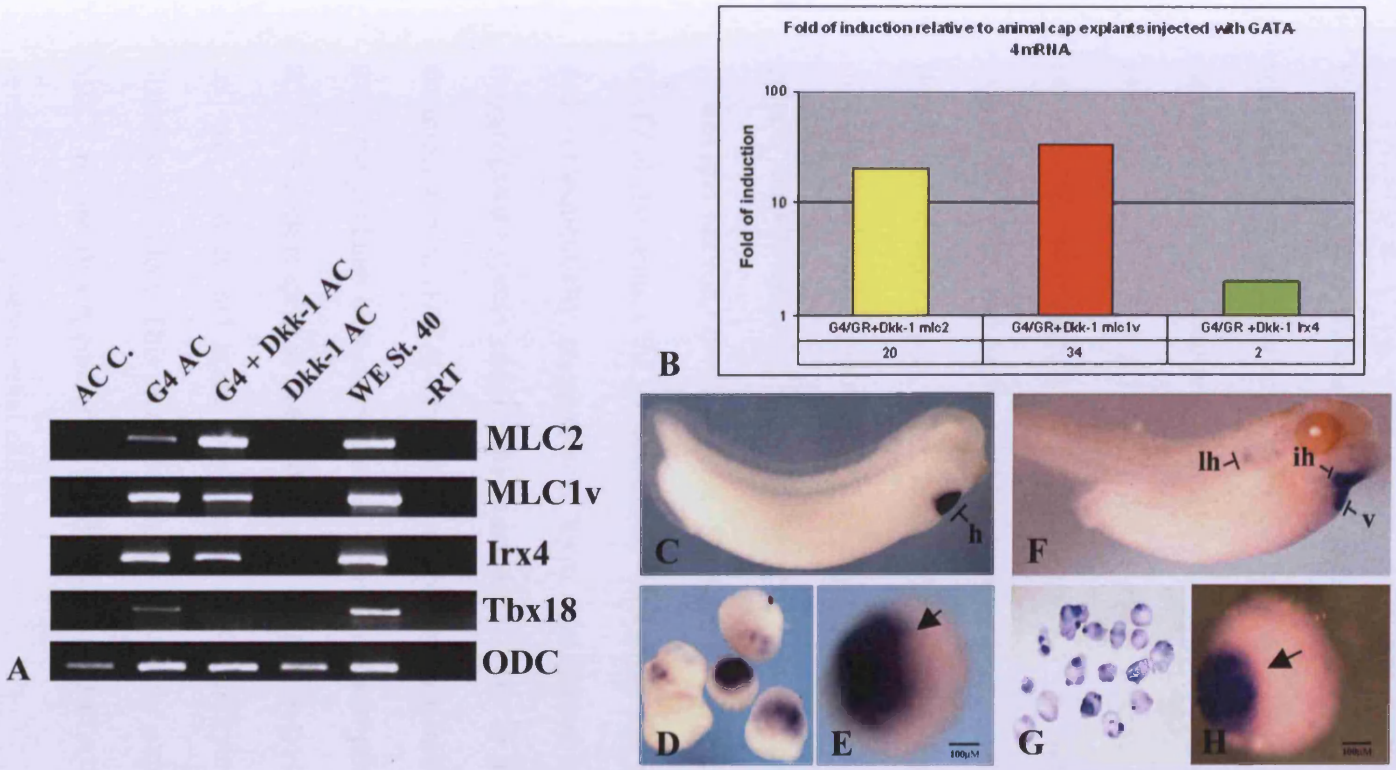

Figure 3.3 Dkk-1 enhances cardiogenic activity of GATA-4 in animal cap explants. (A) Co-injected AC explants show the expression of MLC2, MLC1v and Irx4 when analysed using RT-PCR. (B) Co-injected AC explants analysed using qRT-PCR showed a fold increase of expression of MLC2 and MLC1v when compared to GATA-4 injected explants. (C) Control embryo analysed by WMISH for the expression of MLC2. (D-E) 72% of co-injected AC explants expressed MLC2 (n=33) in WMISH analysis (arrow). (F) Control embryo analysed by WMISH for the expression of MLC1v. (G-H) 50% of co-injected AC explants expressed MLC1v (n=25) when analysed by WMISH (arrow). ih; interhyoid muscles of the jaw; lh; lymph heart, v; ventricle, AC; animal caps control, G4; GATA-4, WE; whole embryos.

3.2.3 Dissociation abolishes ventricular cell fate in GATA-4 injected explants

Expression of pan-myocardial, ventricular and proepicardial markers in AC explants injected with GATA-4 or co-injected with GATA-4 and Dkk-1demonstrates that cell diversification has occurred in the explants. Expression of a pan-myocardial marker depends directly on the injection of GATA-4 mRNA which triggers cardiac induction in the explants. However, cardiac cell diversification in the explants may also depend on the embryonic dorso-ventral axis (D-V) which has been established before the excision of the animal caps from the embryo (De Robertis and Kuroda, 2004; Sokol and Melton, 1991). It has been shown that the capacity of the animal cap explants cells to differentiate into a specific tissue type is related to the inducer activity and also to the differential competence of the cells in the responding tissue (Sokol and Melton, 1991). To test if the pre-patterning in animal cap explants injected with GATA-4 determines the expression of ventricular and proepicardial markers, we dissociated and subsequentially reaggregated them. Uninjected animal cap explants dissociated and reaggregated express endodermal markers (Kuroda *et al.*, 2005) but when the same explants are injected with GATA-4 they express the pan-myocardial marker MLC2 (fig. 3.4). However, the same explants lack expression of ventricular and proepicardial markers when analysed by RT-PCR with respect to injected intact caps (fig. 3.4). Dissociation leads to disruption of the cell-cell contact and upon reaggregation any embryonic patterning established before the dissociation is lost. This result demonstrates that the expression of the pan-myocardial marker MLC2 is directly dependent on the injection of GATA-4 but this is not sufficient to establish ventricular and proepicardial cell fates.

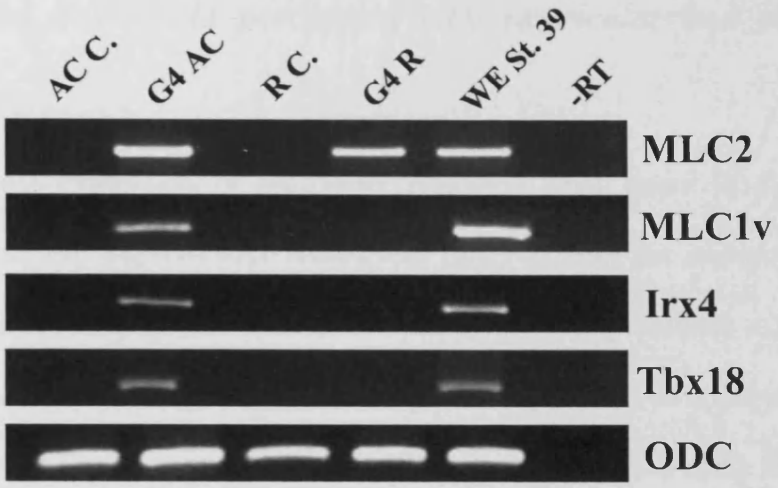

Figure 3.4 Loss of regionally restricted marker expression in dissociated explants. Animal cap explants were dissociated and reaggregated and cultivated until stage 39. Reaggregated explants analysed using RT-PCR showed the expression MLC2 but not the expression of ventricular and proepicardial markers. GATA-4 AC explants express MLC2, MLC1v, Irx4 and Tbx18 when analysed by RT-PCR at the same stage. AC; animal caps control, G4 AC; GATA-4 injected AC explants, G4 R; GATA-4 reaggregated explants, R C; control reaggregated explants, WE; whole embryos.

3.2.4 Dissociated heart field precursors lack ventricular and proepicardial gene expression

The absence of the expression of regionally restricted heart genes in dissociated and reaggregated animal cap explants demonstrate the importance of the embryonic patterning established in the embryo by stage 9. In contrast, pan-myocardial genes such as MLC2 are not affected by dissociation in GATA-4 injected explants (fig. 3.4). An additional method to investigate the mechanism of cardiac gene expression diversification is using heart field (HF) explants. The HFs are able to grow and differentiate autonomously in culture and for this reason can be classified as a morphogenetic field (Raffin *et al.*, 2000). In cultivation they demonstrated the capacity to generate a looped beating structure (Raffin *et al.*, 2000) with a low degree of morphogenesis. Endodermal tissues allow cardiac differentiation to occur in HF explants but it is not sufficient to generate obvious atrium and ventricle chambers (Mohun *et al.*, 2003). The HF was excised from the embryo at stage 21 (fig. 3.5 A), cultivated until stage 40 and analysed by double WMISH for the expression of MLC2 and MLC1v (fig. 3.5 C-F). Double WMISH in the HFs showed a region in which the expression of MLC2 and MLC1v overlapped indicating the position of the ventricle (fig. 3.5 C-F) while the regions that are MLC2 positive only presumably correspond to the atrium (fig. 3.5 C-F). In HF explants ventricular and atrial cell fates exists but the degree of morphogenesis is very low compared to the normal heart. RT-PCR analysis of HFs and HF without endoderm at stage 40 also confirmed the expression of Irx4 and Tbx18. Tbx18 expression is very weak in heart field explants and is almost lost in HF lacking endoderm (fig. 3.5 B). RT-PCR analysis was also carried out in dissociate and reaggregated HFs at stage 40. Those explants showed the expression of MLC2 but not the expression of the ventricular and proepicardial markers (fig.

3.5 B). Expression of MLC2 was lower with respect to the other samples. The HF precursors are probably more dependent on cell-cell contact for the maintenance of cardiac fate compared to GATA-4 reaggregates, in which this is largely a cell-autonomous process.

These results demonstrate that chamber cell fate diversification occurs later than cardiac specification and it requires specific cell-cell contacts to occur. In addition, the results showed that gene expression found in GATA-4 reaggregates closely resembles gene expression found in HF reaggregates as an *in vivo* source of cardiac precursors. Thus, the lacking of ventricular and proepicardial gene expression found in GATA-4 reaggregates is not peculiar but represent a physiologically relevant result.

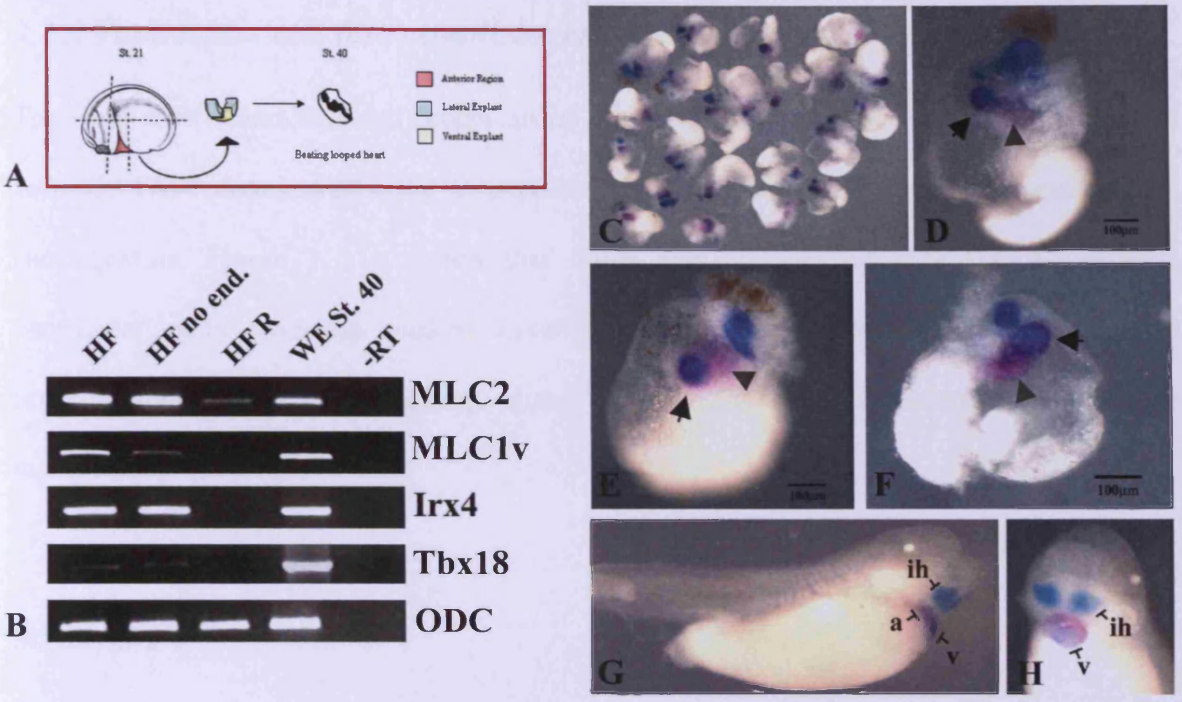

Figure 3.5 Heart field loose ventricular gene expression when dissociated and reaggregated. (A) Heart Field (HF) were excided from embryo at stage 21 and cultivated until stage 40 and (B) analysed by RT-PCR together with HF without endoderm and HF reaggregated at the same stage. (C-F) HF were also analysed at stage 40 by double-WMISH for the expression of MLC2 and MLC1v. WMISH analysis showed regions of overlapping expression between MLC2 and MLC1v corresponding to the ventricle (arrows) and regions MLC2 positive only corresponding to the atrium (head arrows). (G-H) Control embryos at stage 40 were analysed by WMISH for the expression of MLC2 and MLC1v. a; atria, ih; interhyoid muscles of the jaw; r; reaggregate, v; ventricle, HF; heart field, HF no end.; HF without endoderm, HF R; heart field reaggregated, WE; whole embryos.

3.2.5 Ventricular cell fate establishment can be rescued in reaggregated cells

The absence of ventricular and proepicardial gene expression in dissociated explants injected with GATA-4 demonstrates the importance of embryonic pre-patterning in establishing cardiogenesis. Figure 3.1 A shows that intact explants injected with GATA-4 express ventricular and proepicardial markers. In order to re-establish the expression of ventricular and proepicardial markers in dissociated animal cap explants we used molecules known to be involved in patterning the heart.

3.2.5.1 Dkk-1

Dkk-1 enhances cardiogenic activity of GATA-4 in AC explants which show the expression of ventricular and proepicardial markers (fig. 3.3 A-B). In order to verify if it is possible to re-establish cell diversification in dissociated explants, GATA-4 reaggregates were treated with Dkk-1 or XWnt-8 mRNA. We co-injected the reaggreagate with GATA-4 and Dkk-1 to compare the results obtained with the reaggregates injected with GATA-4 (fig. 3.4). Moreover, we combine Dkk-1 injected AC explants at stage 9 with GATA-4 injected reaggregates at the same stage. The aim of this experimental design was to verify if the source of signal provided by Dkk-1 is capable to re-establish the polarity lost in GATA-4 reaggregates by the dissociation process. We also combined XWnt-8 injected AC explants at stage 9 with GATA-4 reaggregates at the same stage. For the same reason using Wnt-8 injected AC (as a source of signal) we aimed to change the A-P polarity in GATA-4 reaggregates and verify if it was possible to re-establish cardiac cell fate diversification.

RT-PCR analysis of the samples at stage 39 showed that MLC1v expression was re-established in GATA-4 and Dkk-1 co-injected reaggregates in contrast to GATA-4 injected reaggregates. The same expression was also found in GATA-4 reaggregates combined with Dkk-1 AC explants (fig. 3.6). GATA-4 reaggregates did not express MLC1v when combined with XWnt-8 injected explants. The same sample showed a low level of MLC2 expression with respect to the other samples (fig. 3.6) confirming that the source of signal provided by XWnt-8 did not re-establish the cardiac cell fate but partially inhibited cardiogenesis. Irx4 expression found in GATA-4 reaggregates combined with Wnt-8 reflects its expression in the head region (Garriock *et al.*, 2001). Therefore, XWnt-8 has most likely neuralised GATA-4 reaggregates.

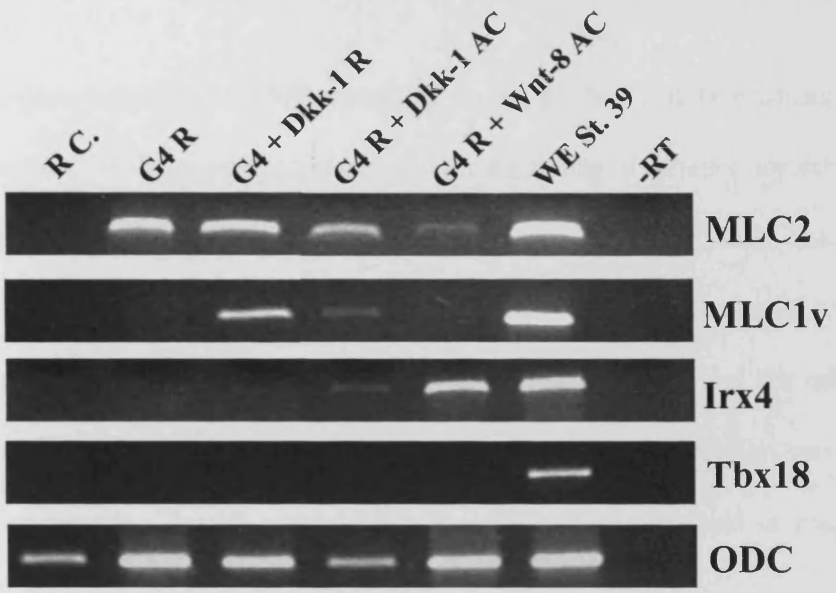

Figure 3.6 Ventricular cell fate can be rescued with Dkk-1 in reaggregates. (A) RT-PCR analysis was performed in explants at stage 39 for the expression of ventricular and proepicardial markers. MLC1v was expressed in GATA-4 reaggregates co-injected with Dkk-1 or combined with Dkk-1 AC. Irx4 was expressed in GATA-4 reaggregates combined with Dkk-1 AC and in GATA-4 reaggregates combined with XWnt-8 AC explants. Tbx18 expression was not rescued. AC; animal caps, R C; reaggregates control, R; reaggregates, WE; whole embryos.

3.2.5.2 BMP

In order to examine the effect of BMP signalling during cardiac cell diversification, GATA-4 reaggregates and AC explants were co-injected with the mutant dominant-negative form of the type I BMP specific receptor, tBr mRNA (Graff *et al.*, 1994). AC explants and reaggregates were cultivated until stage 40 and analysed by RT-PCR for the expression of ventricular and proepicardial markers. Reaggregates co-injected with both GATA-4 and tBr mRNA showed the expression of the ventricular marker MLC1v (fig. 3.7). Irx4 expression was not found in co-injected reaggregates. Tbx-18 expression was only partially rescued in reaggregates co-injected with GATA-4 and tBr mRNA (fig. 3.7).

These results demonstrate that cardiac cell diversification can be restored in GATA-4 reaggregates via the inhibition of BMP pathway or via Dkk-1. Dkk-1 can only rescue the expression of the ventricular markers MLC1v and Irx4 while the inhibition of BMP allows the expression of ventricular and proepicardial genes in reaggregated explants. Thus, ventricular cell fate might be specified by anterior signals (as shown in Dkk-1 and tBr results) while proepicardial cell fate require Wnt signalling, as Dkk-1 inhibits it in intact AC explants.

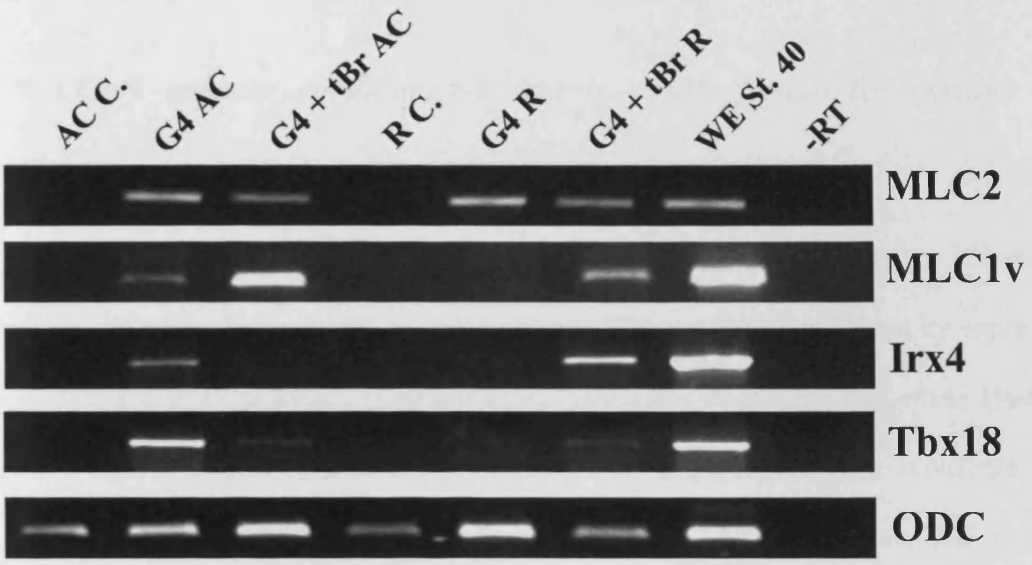

Figure 3.7 Co-injection of GATA-4 with tBr mRNA rescues the ventricular and proepicardial gene expression in reaggregates. GATA-4 reaggregates co-injected with tBr show ventricular and weak proepicardial gene expression with respect to GATA-4 injected reaggregates. Similar expression was found in injected and co-injected intact caps. Expression of Irx4 is lost in co-injected intact caps but no further analysis has been done to explain this result.

3.3 Discussion

3.3.1 GATA-4 induces cardiogenesis but is not sufficient for cardiac cell diversification

Cardiogenesis assay is a simple method to induce cardiac differentiation using animal cap (AC) explants. Cardiac tissue in AC is induced by GATA-4 mRNA, as shown by expression of pan-myocardial markers MLC2 (Latinkic *et al.*, 2003) and cTnI (Drysdale *et al.*, 1994). In this thesis it was shown that GATA4-injected AC explants express ventricular and proepicardial markers when cultivated until stage 40 (fig. 3.1). Moreover, AC explants analysed by double WMISH showed gene expression patterning between the pan-myocardial markers MLC2 and the ventricular marker MLC1v. MLC2 and MLC1v showed overlapping expression in the explants in the region corresponding to the ventricle, while the region where MLC2 was expressed alone is presumed to correspond to the atria (fig. 3.2). The atrial specific marker ANF was not used in WMISH and RT-PCR analysis as it becomes atrial-specific marker only at stage 49 (Small and Krieg, 2000). We found that AC explants do not survive if cultivated until stage 49 and for this reason, and since there are no other suitable atrial markers available in *Xenopus* that we are aware of, we decided to use for RT-PCR and WMISH analysis pan-myocardial, ventricular and proepicardial markers. Expression of MLC2, MLC1v, Irx4 and Tbx18 in AC explants demonstrated that cardiac cell diversification has occurred (fig. 3.1). We have shown that GATA-4 mRNA injection is necessary but not sufficient to allow the AC cells to differentiate in diverse heart cell types. When GATA-4 injected explants were dissociated and reaggregated and analysed at stage 39-40 they expressed MLC2 but not ventricular and proepicardial markers (fig. 3.4). Grunz and Tacke, showed that when uninjected explants were dissociated they lost cell-cell contacts and the

intercellular signals which lead the cells to autonomous neuralisation, when kept dispersed in cultivation for up to 5 h (Grunz and Tacke, 1989). Importantly, the explant cells loose any embryonic pre-patterning (regional differences) previously established. It has been shown that cells in intact AC explants are not uniform but have a dorsoventral polarity. In fact, when the prospective dorsal and ventral regions of AC explants were dissected and treated with activin they differentiated into dorsoanterior and ventrolateral structures respectively (Sokol and Melton, 1991). This demonstrated that the capacity of the explants to express ventricular and proepicardial markers depends on GATA-4, but also on a pre-existing pattern in the explants. Thus, the lack of cardiac cell diversification in dissociated GATA4-injected AC explants results from the loss of cell-cell contact and the loss of embryonic pre-patterning. Similarly to GATA-4 reaggregated explants, heart field explants (HF) dissociated and reaggregated express MLC2 but lack of ventricular and proepicardial markers (fig. 3.5). The loss of cell-cell contact during neurula stage (stage 20) demonstrated that the cardiac cell diversification occurs later that this developmental stage. Intact HF cultivated until stage 39 express ventricular and proepicardial markers and when analysed in WMISH showed patterning of expression between MLC2 and MLC1v (fig. 3.5 C-F).

3.3.2 Dkk-1 enhances cardiogenesis in AC explants

It has been shown that Dkk-1 is a Wnt/β-catenin antagonist (Semenov *et al.*, 2001) and acts as head inducer via the anteriosation of the body structure during embryo development (Glinka *et al.*, 1998). When Dkk-1 is co-injected with GATA-4 mRNA it enhances cardiogenesis in AC explants (Latinkic *et al.*, 2003). Co-injected AC explants showed the expression of pan-

myocardial marker MLC2 and also the expression of ventricular markers MLC1v and Irx4 (fig. 3.3). Quantitative analysis (qRT-PCR, fig. 3.3 B) demonstrated that the co-injection of GATA-4 with Dkk-1 enhances the expression of MLC2, MLC1v and Irx4 when compared with the expression of the same markers in GATA-4 injected explants. Thus, in AC explants Dkk-1 increases the expression of MLC2 that most likely accounts for the increase of MLC1v. Moreover, we believe that the enhancement of MLC1v can be also related with the capacity of Dkk-1 to anteriorise the body structure during embryos development (Glinka *et al.*, 1998). During the heart tube stage the cell diversification has already occurred along the A-P axis and the ventricle cells are specified more anteriorly compared to the future posterior location in adult heart. We showed that MLC1v is expressed at high levels in co-injected AC explants compared to Irx4 expression (fig. 3.3). This is related with the embryonic expression of Irx4 in the ventricle which is lower that MLC1v (Garriock *et al.*, 2001; Smith *et al.*, 2005). GATA-4 injected AC explants showed the expression of Tbx18 which is a proepicardial marker (Jahr *et al.*, 2008). The expression of Tbx18 is a good indicator of cardiac cell diversification because it demonstrated that GATA4-injected AC explants are composed of myocardial and non-myocardial cardiac cells. It has been shown that cardiac progenitors positive for expression of Nkx 2.5 and Isl-1 contribute to the development of the proepicardium (Zhou *et al.*, 2008). In fact, cells expressing the proepicardium marker Wt-1 (Perez-Pomares *et al.*, 2009) are descendent from the cardiac precursors which express both Nkx 2.5 and Isl-1 (Zhou *et al.*, 2008). In AC explants co-injected with GATA4 and Dkk-1 the expression of Tbx18 is absent (fig. 3.3), leading us to conclude that Dkk-1 expression in AC inhibits Tbx-18 during the development of the epicardium. The role of Dkk-1 in epicardium differentiation has been investigated in mutant mice null for Dkk-1 and Dkk-2 (Phillips *et al.*, 2010). Inhibition of Dkk-1 generates a multilayered epicardium which is due to an over-specification of

proepicardial cells rather that increased proliferation of the mature epicardium. Thus, it is believed that Wnt signalling plays a positive role in specifying the proepicardium (Phillips *et al.*).

3.3.3 Ventricular expression can be rescued in reaggregated explants

GATA-4 dissociated and reaggregated AC explants do not express ventricular markers as in intact AC explants. This expression however can be rescued by manipulating the explants with signalling pathways known to be involved in heart development. In particular, Dkk-1 showed the capacity to restore the ventricular gene expression in those reaggregates. GATA-4 and Dkk-1 co-injected reaggregates showed the expression of MLC1v in contrast to GATA-4 reaggregates. MLC1v expression was also found in GATA-4 reaggregates combined with Dkk-1 AC (fig. 3.6). Therefore, we believe that the re-establishment of the ventricular gene expression in these reaggregates depends directly with the anterior signal provided by Dkk-1. Irx4 expression was found in GATA-4 reaggregates combined with Dkk-1 AC and also in GATA-4 reaggregates combined with Wnt-8 AC (fig. 3.6). We believe that the expression found in reaggregates combined with Dkk-1 is related to the ventricular expression, while the expression found in GATA-4 reaggregates combined with Wnt-8 AC is related with the expression of this marker in the head regions of the embryo (Garriock *et al.*, 2001). Thus, we believe that XWnt-8 AC has neuralised GATA-4 reaggregates. This may also explain the low level of MLC2 expression found in the same reaggregates. Tbx18 was not expressed in these samples. This is probably dependent on the direct negative effect of Dkk-1 on the epicardium specification (Phillips *et al.*). Similarly to Dkk-1 also BMP plays an important role during

cardiogenesis. We have shown that inhibition of BMP via co-injection of tBr, mutant form of the type I BMP specific receptor which inhibits BMP signalling, with GATA-4 in reaggregates can restore the expression of MLC1v, Irx4 and unlike Dkk-1, also Tbx18 (fig. 3.7). During early *Xenopus* embryo development the inhibition of BMP-4 generates the conversion of the ventral mesoderm to the dorsal mesoderm (Graff *et al.*, 1994). During gastrulation ventricular myocardial progenitor cells show a dorsal location along the embryonic dorsoventral axis (Keegan *et al.*, 2004) corresponding with the level of FGF (Marques and Yelon, 2009). Inhibition of BMP and the conversion of ventral mesoderm to dorsal mesoderm (Graff *et al.*, 1994) may lead to an increase of ventricular cell fate in the reaggregated explants. As described in zebrafish, BMP regulates the cardiac chamber proportion (Marques and Yelon, 2009). Overexpression of BMP signalling generates ventralised embryos which displayed an enlarged atrium and an increased number of atrial cardiomyocites (Marques and Yelon, 2009). This evidence opens an opportunity to study the role of this signalling pathway in the regulation of the cell fate diversification during cardiac differentiation. In addition, Glinka and colleagues, showed that the inhibition of BMP and Wnt leads in whole embryos the induction of the head structure during gastrula stage in the endomesoderm (Glinka *et al.*, 1997). We believe therefore that the inhibition of BMP may share the mechanism with Dkk-1 in inducing ventricular expression in reaggregated explants. Both Dkk-1 and tBr probably enhance anterior identity in the reaggregated GATA4-expressing explants, thereby specify ventricular cell fate. Co-injection of tBr and GATA-4 in reaggregates partially rescues the expression of the proepicardial marker Tbx18 (fig. 3.7). Schlueter and colleagues, showed in chick that both BMP2 and BMP4, which are expressed in the sinus venus myocardium, may contribute to the recruitment of the LPM cells to the proepicardium cell lineage. When proepicardium explants were treated with BMP2 or BMP antagonist Noggin they both resulted in a loss of

proepicardium markers Tbx18, Wt1 and Cfc. Moreover, the same explants showed an upregulation of myocardial markers (Schlueter *et al.*, 2006). In contrast to the results obtained in chick, recent evidence in zebrafish showed that BMP together with Tbx5 are essential for proepicardium specification (Liu and Stainier, 2010). Mutant zebrafish lacking of BMP4 had reduced level of Tbx18 and tcf21 proepicardial markers. Moreover, BMP2 overexpression induced an ectopic overexpression of Tbx18 but not tcf21 indicating that BMP signalling alone is not sufficient to completely commit cells to the proepicardial fate (Liu and Stainier, 2010). Recent evidence showed also that GATA-4 play an important role in proepicardial specification. In mice GATA-4 is highly expressed in the proepicardium and GATA-4 null mice lacked completely the proepicardium cells (Watt *et al.*, 2004). Furthermore, evidence showed that GATA-4 acts as downstream target of BMP4 in lateral mesoderm (Rojas *et al.*, 2005). These results together with the results of Liu and Stainier are consistent with a model in which BMP4 regulates the proepicardium specification via the expression of GATA-4 (Svensson). The results found in GATA-4 and tBr co-injected rearregates and AC explants (fig. 3.7) are more consistent with those found in zebrafish (Liu and Stainier, 2010). The low level of expression of Tbx18 may be directly related with the inhibition of BMP signalling. However, we are not capable to explain the lack of Irx-4 expression in co-injected AC explants in which we expected the expression of this marker as inhibition of BMP causes dorsalised embryos (Graff *et al.*, 1994). Further investigation is required to understand how Dkk-1 and BMP regulate cardiac cell fate diversification in these explants.

3.3.4 Ventricular cell fate specification occurs after neurula stage

In parallel to AC explants assay we used also heart field (HF) precursors excised from whole embryos. HFs are very useful to understand how the cardiac cell diversification is established during heart development. HFs can be considered as morphogenic field for the capacity to grow a beating structure when excised from the embryo and cultivated until late developmental stages (Raffin *et al.*, 2000). HFs in isolation expressed pan-myocardial marker MLC2, ventricular markers MLC1v and Irx4 and also proepicardial marker Tbx18 (fig. 3.5). Moreover, they beat but the heart tube shows an incomplete looping. In contrast, HFs dissociated and reaggregated at stage 21 and cultivated until stage 40 expressed MLC2 but lacked MLC1v, Irx4 and Tbx18 gene expression (fig. 3.5 A). Thus, they behave as GATA-4 reaggregates which do not show ventricular and proepicardial cells diversification. The inducing activity to specify the cardiac precursor has occurred as the reaggregated HFs express MLC2 but the dissociation and reaggregation during neurula stage showed that the cell diversification has not yet been determined and occurs prior the beginning of the heart differentiation.

4. *Analysis of morphogenesis in explants transplanted into Xenopus host embryos*

4.0 Analysis of morphogenesis in explants transplanted into *Xenopus* host embryo

4.1 Introduction

In the previous chapter we have shown that in animal cap explants GATA-4 induces the expression of pan-myocardial gene MLC2, the ventricular markers MLC1v and IRX4 and the proepicardial marker Tbx18. This gene expression depends on the injection of the cardiac inducer GATA-4 and also on intact AC tissue integrity, possibly reflecting the requirement for pre-patterning in the explants. This confers to the caps the capacity to generate a simple structure which at late developmental stages is capable of beating. However, the beating activity is rare and is usually associated with morphologically non-distinct tissue.

To understand how rudimentary heart morphogenesis is established we required a reliable and simple assay. This was provided by modifying and combining two assays: 1) GATA4-injected animal caps (described in Ch. 3) and 2) secondary heart formation assay (Ariizumi *et al.*, 2003). As described previously, when animal cap explants were dissociated, reaggregated and cultivated they express pan-myocardial marker MLC2 but completely lack ventricular marker expression. It is likely that the absence of ventricular and proepicardial markers is caused by the dissociation process which disrupts the cell-cell contact and the embryonic patterning previously established. Ariizumi and colleagues obtained a secondary heart inside a *Xenopus* host embryos using activin induced reaggregated animal caps. When transplanted in host embryos, reaggregated cells are able to generate a complex structure capable of beating (Ariizumi *et al.*, 2003). We found that the use of GATA-4 injected reaggregates was more reliable that the use of reaggregates treated with activin. For this reason, we performed our experiments using GATA-4

mRNA. We confirmed the ability to generate a secondary beating heart-like structure from GATA-4 reaggregates transplanted into *Xenopus* host embryos. The *in vivo* heart formation assay is a useful method of re-establishing the gene expression lost in cultivated reaggregate explants and is also a functional assay to study the emergence of basic heart morphogenesis, growth and physiology.

4.1.1 Chapter Aim

Using GATA-4 injected explants, we aimed to study:

1. The generation of a simple and reliable assay to study heart patterning and morphogenesis after cardiac specification.
2. The role of the host in the generation of a secondary heart-like structure.

4.2 Results

4.2.1 GATA-4 injected reaggreagates generate a secondary beating heart when transplanted into a host embryo

Heart development involves a complex series of steps including specification of regionally restricted cell types, growth and looping which ultimately results in emergence of embryonic heart composed from two atria, one ventricle and OFT. Whilst the animal cap assay is powerful for the investigation of cardiac specification, in our experience it does not support more advanced steps of cardiogenesis. To study these, we have developed a heart formation assay (HFA). In the HFA, dissociated and reaggregated GATA-4 injected animal cap explants (instead of activin treated ACs used by Ariizumi *et al.*, 2003) were transplanted into host embryos at stage 20 (Ariizumi *et al.*, 2003). The transplants were performed as described in the methods by making a small incision in the ventral side the host where the reaggregate was placed. The use of CAG-GFP transgenic reaggregates and host embryos facilitated the detection of cardiac induction and morphogenesis in the same transplants.

46% of the reaggregates transplanted into host embryos showed beating activity when analysed at stage 40 (fig. 4.1; movie 1). Co-injection of GATA-4 with Rhodamine- and Biotin-dextran helped to confirm the presence of the reaggregate inside the host embryo (fig. 4.1 D). When transplants were carried out using uninjected reaggregates (fig. 4.1 H-I) or reaggregates not treated with dexamethasone (fig. 4.1 J-K) no cardiac induction or beating activity were detected in the transplants.

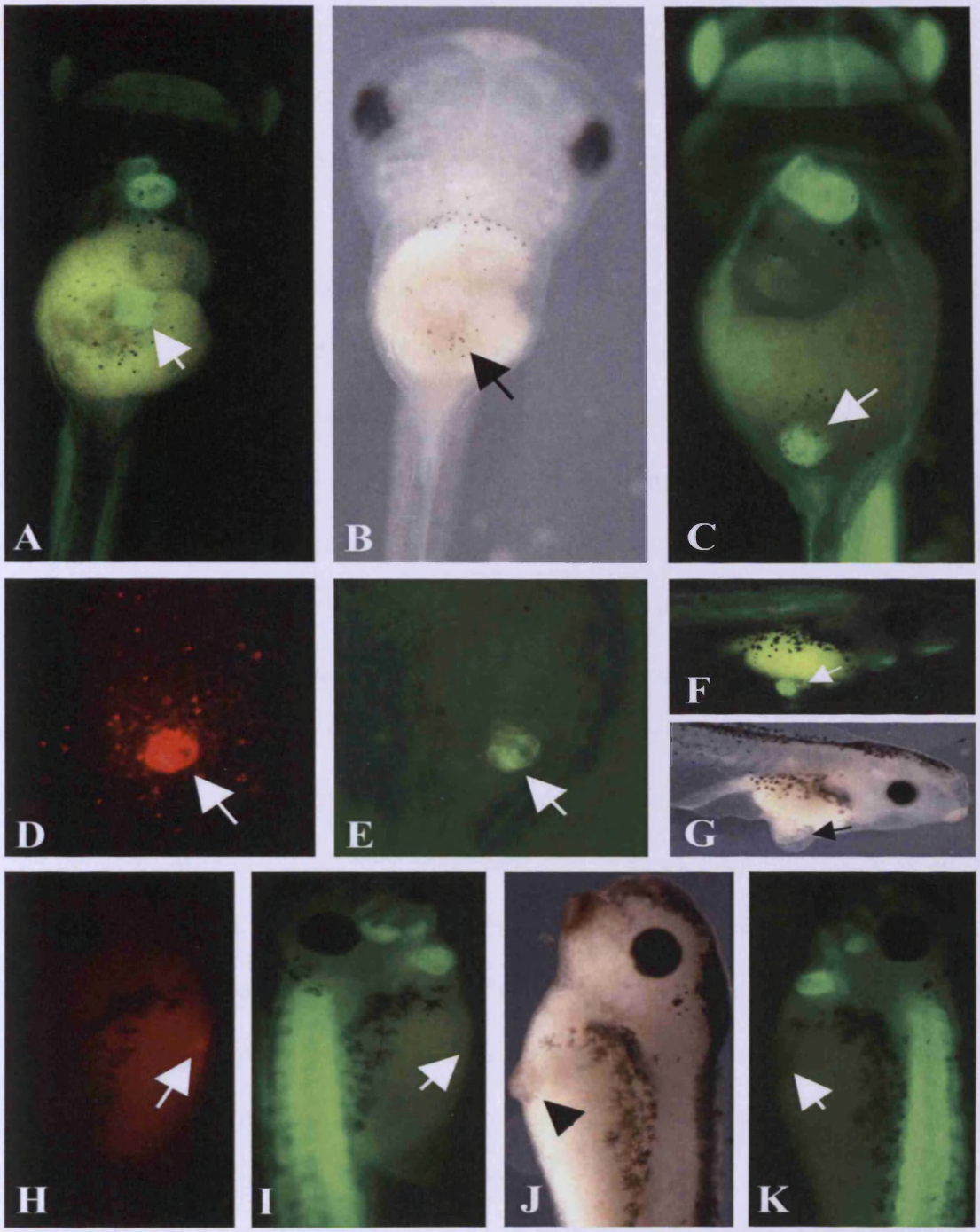

Figure 4.1 46% of GATA-4 injected reaggregates beat when transplanted in host embryos. (A-G) 46% of regaggregates transplanted in host embryos (arrows) showed beating activity in (n=220) when analysed at stage 40. (D) Co-injection with lineage tracer helped to locate the reaggregate inside the host during fluorescent microscope analysis (arrows). (H-I) Reaggregates not treated with Dexametasone (arrows) or (J-K) not injected with GATA-4 mRNA (arrows) did not show any GFP or beating activity.

4.2.1.1 Patterning analysis of the secondary heart

A useful method to detect ventricle-restricted gene expression in reaggregates involves the use of transgenic embryos containing the MLC1v-GFP reporter gene (Smith *et al.*, 2005). Reaggregates (MLC1v-GFP) transplanted at stage 20 into the host embryos at the same stage were positive for GFP expression and showed beating activity when analysed at stage 40 (fig. 4.2). We investigated then the regional distribution of gene expression within the transplanted reaggregates using WMISH analysis. Double-WMISH was performed in transplants at developmental stage 42 for the expression of the pan-myocardial marker MLC2 and the ventricular marker MLC1v (fig. 4.3 A-D). The region of overlapping expression between MLC2 and MLC1v correspond to the ventricle region while the region positive to MLC2 expression alone correspond to the atria (fig. 4.3 A1-C1). 48% of the reaggregates showed distinct ventricle and atria gene expression (fig. 4.3 A1-C1). 17% of reaggregates show only an overlapping expression and 30% of reaggregates exclusively show the expression of MLC2 only suggesting that in those cases incomplete or aberrant development occurred. Reaggregates which showed both clear atrial and ventricle regional gene expression did not show any evidence of alignment with the anterior-posterior (A-P) gene expression patterning of those gene in the heart of the host embryos (fig 4.3 A-C). This result suggests that ventricular and atrial cell fates are not specified by a mechanism that is directly related to A-P axis of the embryo.

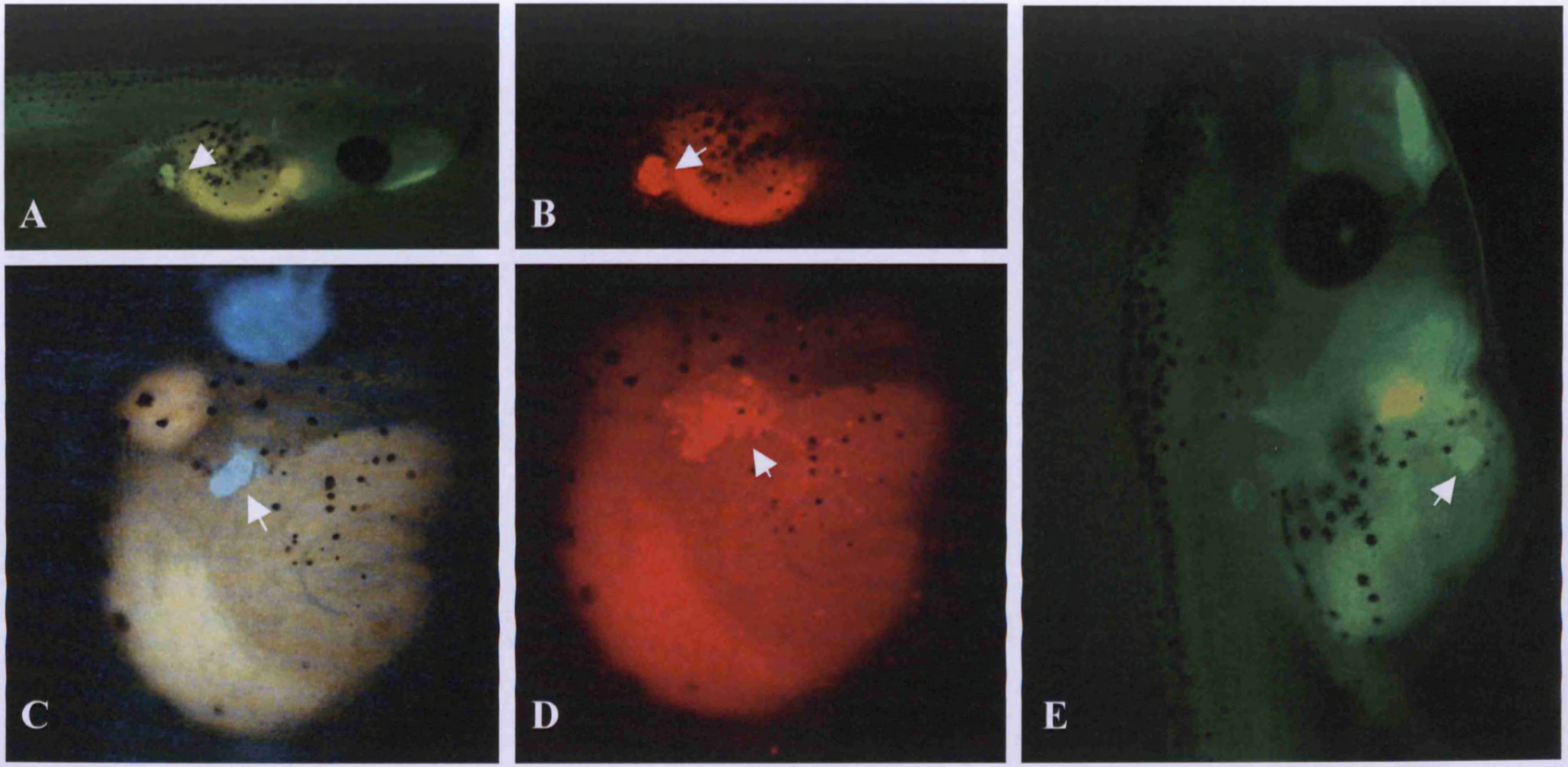

Figure 4.2 GATA-4 reaggregates express MLC1v when transplanted in host embryos. (A-E) MLC1v-GFP transgenic reaggregates (arrows) were previously injected with GATA-4 and transplanted into MLC1v transgenic hosts at stage 20. Reaggregates analysed at stage 40 were positive for GFP expression and showed beating activity.

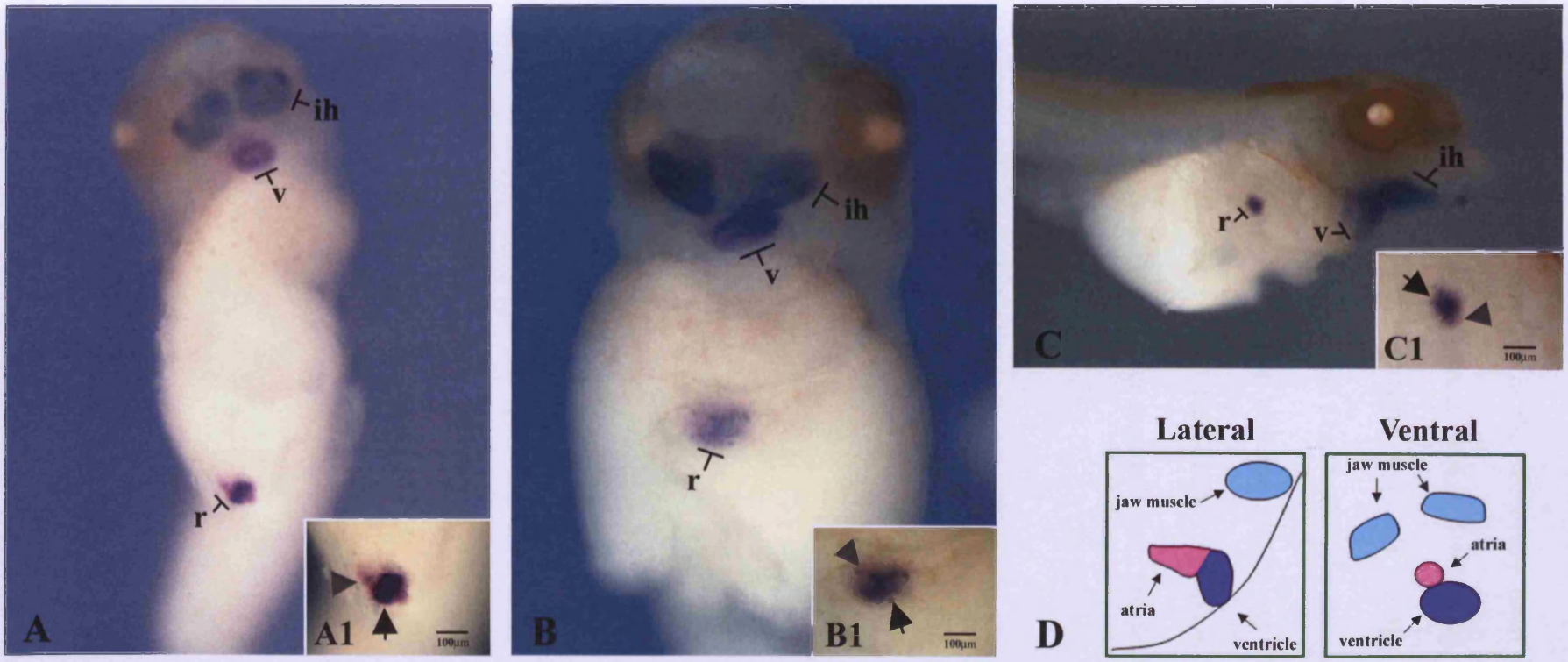

Figure 4.3 48% of transplanted reaggregates show distinct atrial and ventricular gene expression. (A-C) Transplants were analysed using WMISH for the expression of MLC2 and MLC1v at developmental stage 40. (A1-C1) Reaggregates show overlapping expression of MLC2 and MLC1v (48% n=45) (arrow) in the region corresponding to the ventricle and MLC2 expression alone (head arrow) in the atrial region. (D) Double WMISH in heart shows the overlapping expression between MLC1v and MLC2 in the ventricle and expression of MLC2 alone in the atria. MLC1v is also expressed in the jaw muscle. ih; interhyoid muscles of the jaw; r; reaggregate, v; ventricle.

Our analysis of patterning in the secondary heart was extended by WMISH for the expression of the proepicardial marker Tbx18 (fig. 4.4 F). 25 % of the reaggregates analysed using double WMISH (fig. 4.4; n=30) showed an apparent overlapping expression between MLC2 and Tbx18 (fig 4.4 A2) while 40% of the reaggregates showed distinct regions of expression between MLC2 and Tbx18 (fig. 4.4 B1). The transplants therefore developed a completed structure composed of myocardial and non-myocardial cells which express a distinct regional gene distribution capable to proceed toward the physiological step of beating.

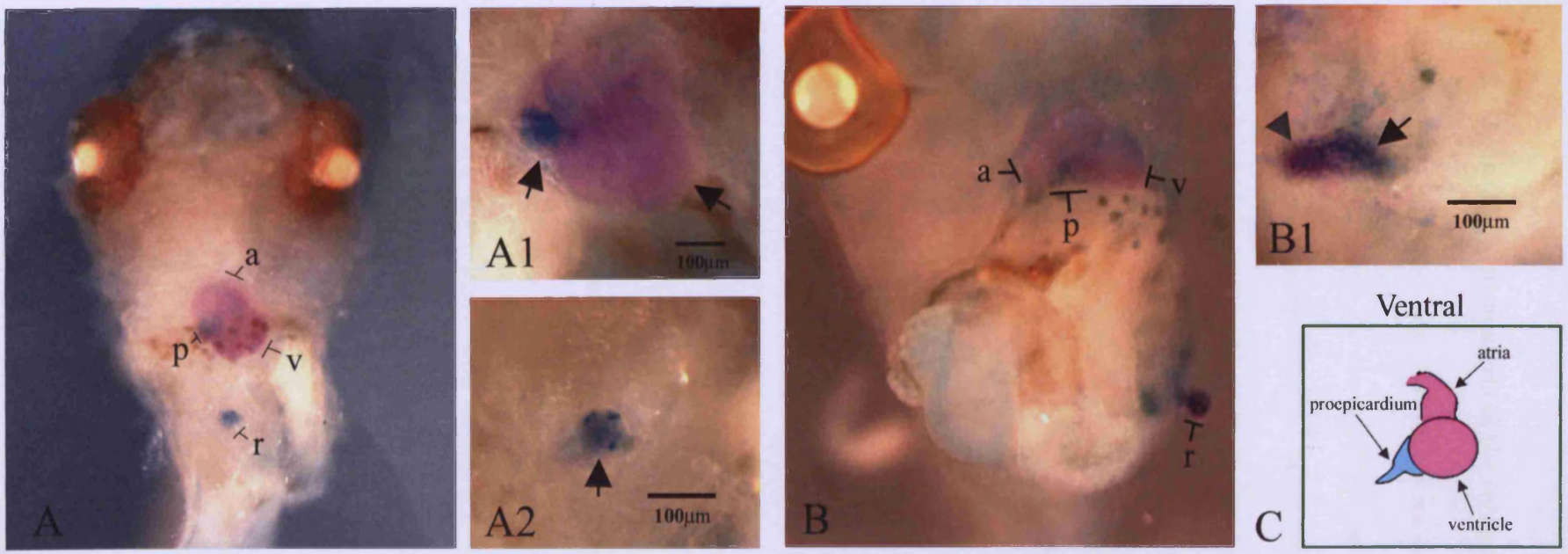

Figure 4.4 Transplanted reaggregates show the expression of proepicardial marker Tbx18. (A-B) Reaggregates transplanted at stage 20 in host embryos at the same stage were analysed by WMISH at stage 40 for the expression of MLC2 and Tbx18 (n=30). (A1) The host heart express Tbx18 in the proepicardium and MLC2 in the heart (arrows). (A2) 25% of the reaggregates show an apparent overlapping expression between MLC2 and Tbx18 (arrow). (B1) 40% of the reaggregates show distinct expression between MLC2 (head arrow) and Tbx18 (arrow). (C) Tbx-18 is proepicardial marker and is localised in the right sinus horn of the heart and it does not have overlapping expression in the heart when is co-analysed with the pan-myocardial marker MLC2.a; atrium, pe; proepicardium, r; reaggregate, v; ventricle.

4.2.1.2 Morphogenic analysis of the secondary heart

Transgenic embryos usage has been very helpful in the detection of cardiac induction in reaggregates and also in the classification of the structure of the beating reaggregates. However, better characterisation of the morphogenesis in the SH required a method capable of higher resolution and this was provided by confocal microscopy. Reaggregates transplanted at stage 20 into host embryos at the same stage were analysed by whole mount immunohystochemestry at stage 42 using a monoclonal antibody raised against cardiac troponin T (Ch-1) (Kolker *et al.*, 2000). The reaggregates (n=43) were excised from the host embryos and analysed by confocal microscopy. Confocal analysis showed two different types of morphogenesis. The first, a tubular structure (34% of reaggregates) in which the degree of morphogenesis indicates a shape bearing a degree of similarity to the heart (fig. 4.5 A). The second type of reaggregates showed (65%) no obvious sign of morphogenesis (fig. 4.5 B-D). Transplanted reaggregates had an apparent synchronised beating activity in which the cells generated a wave. However, beating activity observed in the transplants was not documented using physiological methods.

Taken together these results demonstrate the possibility of obtaining a SH from a group of cells previously injected with GATA-4 mRNA. The SH have distinct cell diversification with regional expression of ventricular and proepicardial markers and beating activity. These findings therefore raised the question of the nature of the host-transplant interactions that are required for SH development.

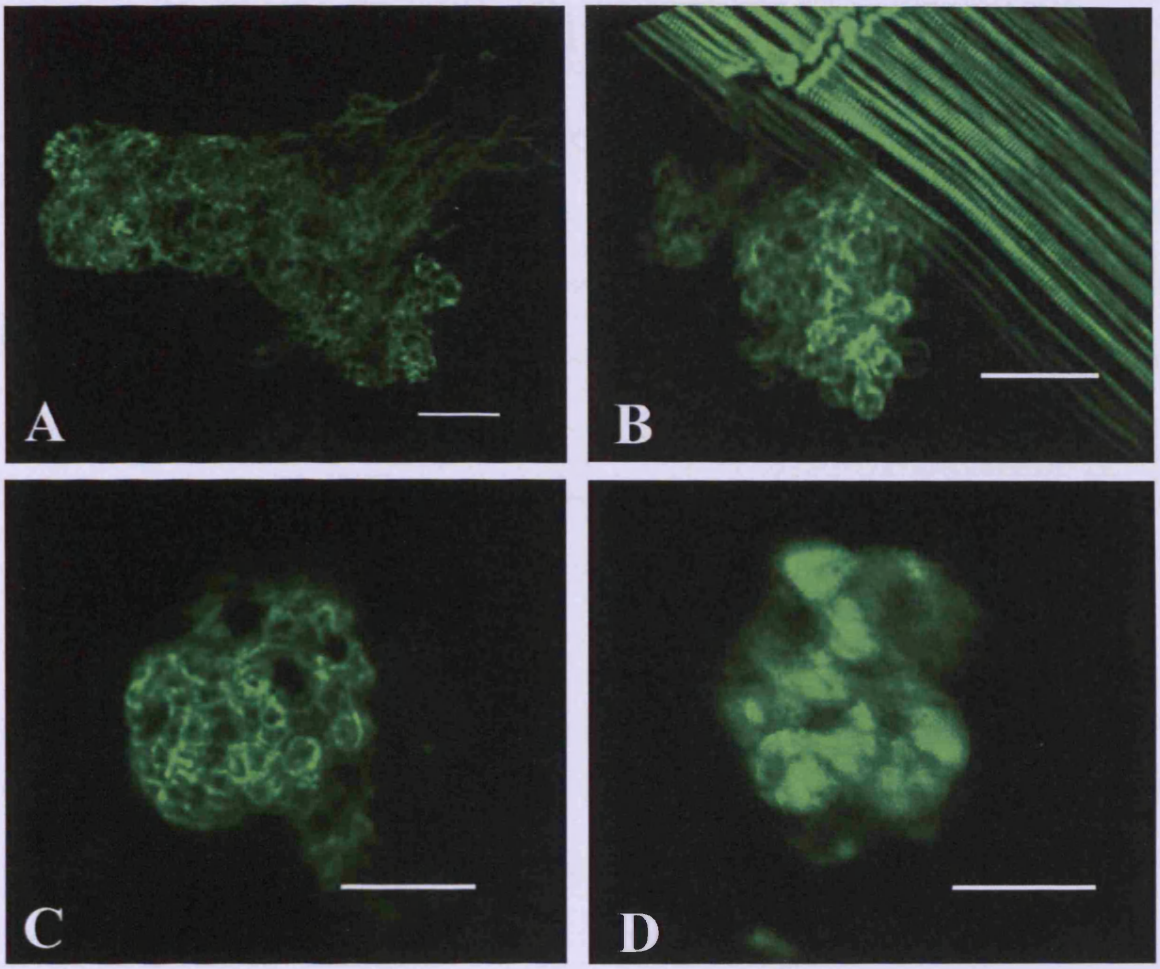

Figure 4.5 Reaggregates show the development of different types of morphogenesis. (A-F) Reaggregates transplanted at stage 20 into host at the same stage were analysed by IMHC. Reaggregates show different degrees of morphogenesis which include (A) a simple tubular structure or (B-F) rudimental group of cell without any significance morphogenesis. The scale bar corresponds to 100μm.

4.2.2 Secondary heart development is lost in UV treated hosts

To further our understanding of SH formation we altered the nature of the transplant-host interactions using UV treatment of host embryos.

UV-treated embryos lack the dorsoventral axis because the exposure to the UV irradiation disrupts microtubule movement and the migration of the dorsal determinants. Therefore, the embryos are completely ventralised. Embryos transgenic for the CAG-GFP reporter were exposed to the UV-light for 30 seconds (Sive *et al.*, 2000) and used as hosts for transplantation at stage 20 with GATA-4 injected reaggregates (CAG-GFP) at the same stage (fig. 4.6 C). Reaggregates transplanted into UV-treated host showed the existence of cardiac induction when analysed at stage 35 (fig. 4.6 D-E). This was confirmed using WMISH analysis for the expression of MLC2 showing that cardiac induction triggered by GATA-4 injection was not affected by UV-ventralisation of the host (fig. 4.6 G-I as indicated by the arrows). Significantly, reaggregates transplanted into UV-treated hosts did not show beating activity and also failed to express ventricular marker MLC1v when analysed at stage 39 using double WMISH for the expression of MLC2 and MLC1v (fig. 4.7 F-G).

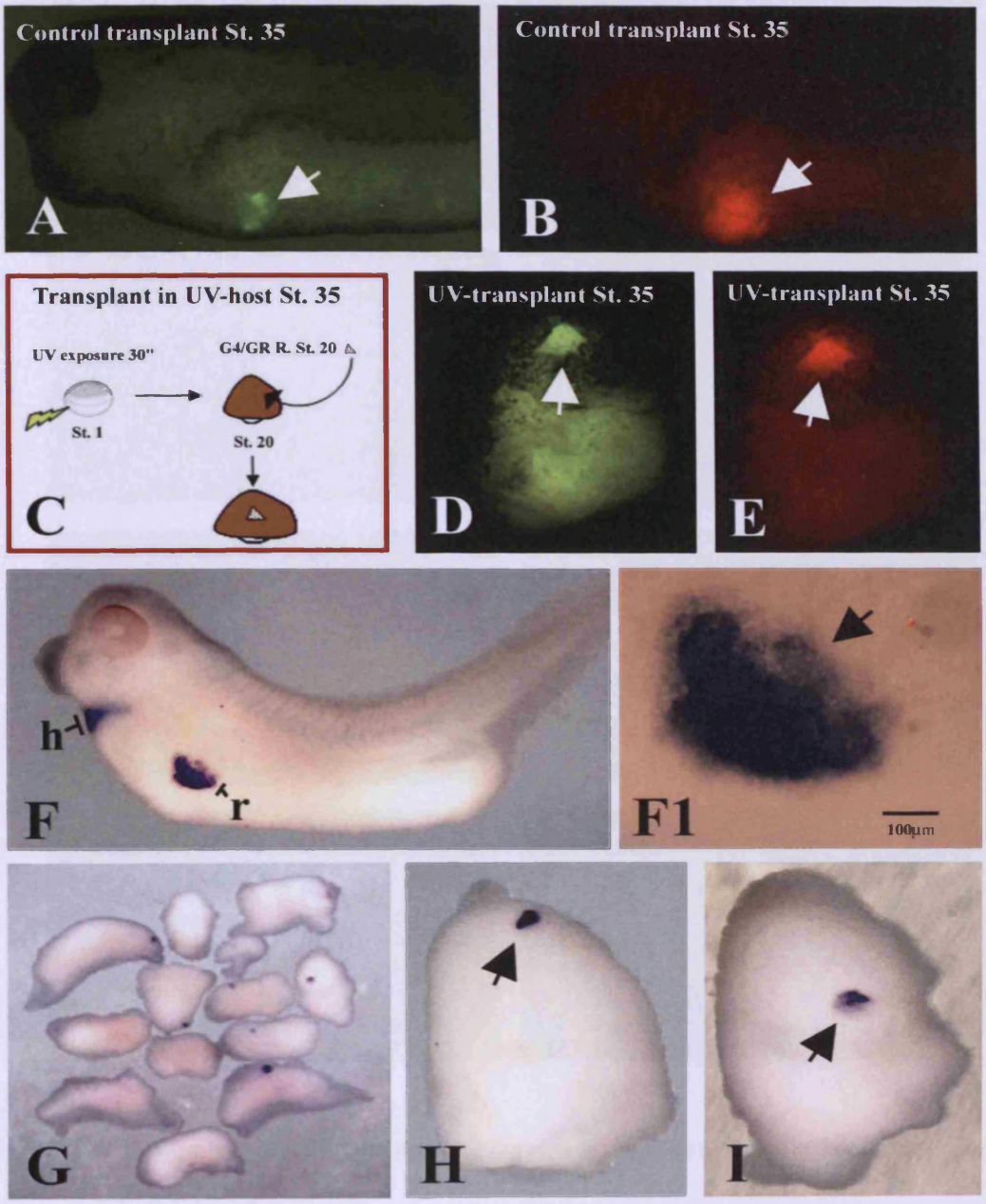

Figure 4.6 Cardiac induction is maintained in reaggregates transplanted into UV-treated host. (A-B) Reaggregates transplanted at stage 20 (arrows) into hosts at the same stage were positive for GFP expression when analysed at stage 35. (C-E) Reaggregates (CAG-GFP) (arrows) were transplanted at stage 20 into UV-treated host at the same stage. GFP expression analysis confirmed cardiac induction. (F-F1) Control transplants at stage 35 were analysed by WMISH for the expression of MLC2 (arrow). (G-I) Reaggregates transplanted into UV-treated hosts (50% n=30) showed the expression of MLC2 (arrows) by WMISH analysis. h; heart, r; reaggregate.

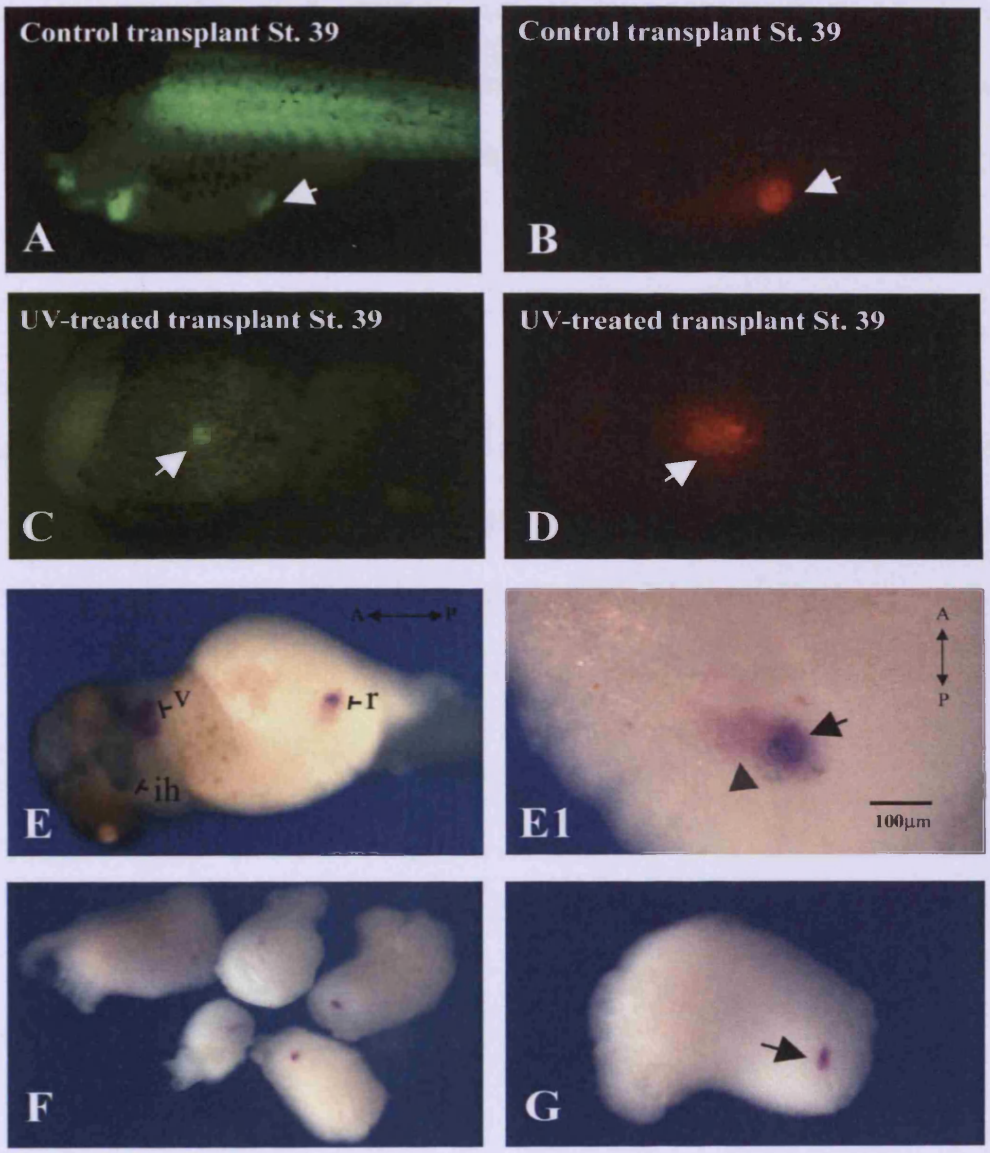

Figure 4.7 MLC1v expression is lost in reaggregates transplanted into a UV-treated host. (A-B) Reaggregates (CAG-GFP) transplanted at stage 20 into hosts at the same stage were positive for GFP expression (arrows) when analysed at stage 39. (C-D) Reaggregates (CAG-GFP) transplanted at stage 20 into UV-treated host at the same stage were positive for GFP expression when analysed at stage 39 (arrows). (E-E1) Control transplants analysed by WMISH showed overlapping regions between MLC2 and MLC1v (arrow) and regions positive for MLC2 only (head arrow). (F-G) Reaggregates transplanted into UV-treated host (35% n=65) showed the expression of MLC2 only when analysed by double WMISH. ih; interhyoid muscles of the jaw; r; reaggregate, v; ventricle. Anterior (A)-posterior (P) axis is indicated.

In addition, UV-treated hosts also failed to support the growth of the reaggregates. UV-treated transplants and control transplants were analysed using fluorescent microscopy for the expression of cardiac actin gene. The 2D analysis suggests that reaggregates transplanted into ventralised UV-hosts (80% n=40) were 40% smaller with respect to reaggregates transplanted into control hosts (fig. 4.8 A1-B1).

These results demonstrate that secondary heart formation (beating, restricted gene expression and growth) requires the host embryonic axes (A-P, D-V). This requirement is unlikely to be direct or instructive because SH polarity is independent from A-P host axis and in addition a wide range of host locations support SH formation (section 4.2.3). For these reasons it is more likely that the signal from the host is permissive for the formation of SH.

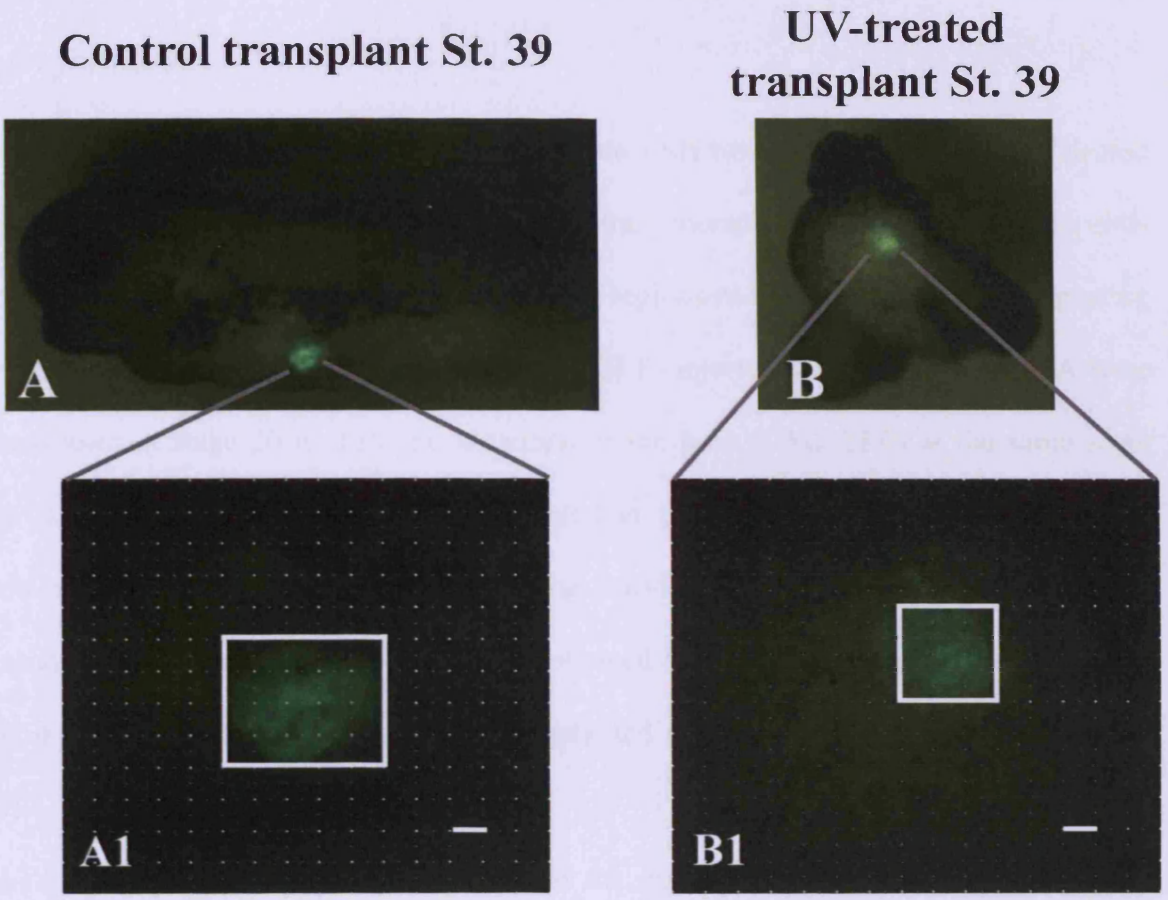

Figure 4.8 UV-treated host does not support the growth of the reaggregate. (A) Control transplants (n=40) and (B) UV-treated transplants (n=40) were analysed at stage 39 by the fluorescent microscope. Reaggregates (CAG-GFP) were positive for GFP expression. (A1-B1) The 2D analysis showed that UV-treated host embryos (80%) does not support the growth of the transplanted reaggregate which has a smaller size when compared with reaggregate transplanted into control host embryo. The bar corresponds to 100μm.

4.2.3 A wide range of permissive regions in the host supports secondary heart formation

Previous results demonstrate the ability to generate a SH from a group of GATA-4 injected reaggregates transplanted into host embryos. As the generation of a functional SH depends on the host, we wanted to identify the region or regions in the host capable of supporting the development of a SH. Reaggregates (CAG-GFP) injected with GATA-4 mRNA were transplanted at stage 20 in different locations of the host (CAG-GFP) at the same stage (fig. 4.9 A). 51% of reaggregates transplanted in the head region of the hosts (n=20) showed cardiac induction but not any beating activity (fig. 4.9 B-C). Transplants in the ventral, lateral and caudal region of the host showed cardiac induction and beating activity (fig. 4.9 D-F) similarly to reaggregates transplanted in the ventral region of the host (fig. 4.1).

GATA-4 injected (CAG-GFP) transplants in the dorsal side of the host lacked cardiac induction when analysed at stage 37 (100% n=20) with respect to control transplants at the same stage (fig. 4.10 C-D and A-B). When the same transplants were analysed at stage 42 they showed cardiac induction (50% n=20) but did not show any beating activity (4.10 E-F). Transplants were also analysed using double WMISH for the expression of MLC2 and MLC1v (fig 4.10 G-G1). The heart of the host showed an overlapping expression in the ventricle region given by the expression of both the markers (fig. 4.10 G). However, 20% of reaggregates in the dorsal region (n=94) express MLC2 and a small region of overlapping expression between MLC2 and MLC1v (fig. 4.10 G1) in contrast to expression pattern found in control reaggregates.

Delay in the expression of GFP in reaggregates transplanted into the dorsal region of the host may be influenced by Wnt/β-catenin signal which is known to be a negative regulator of cardiogenesis (Tzahor and Lassar, 2001). A simple way to verify this hypothesis

involves the use of the Wnt- β catenin pathway inhibitor Dkk-1. Reaggregates (CAG-GFP) co-injected with GATA-4 and Dkk-1 mRNA were transplanted at stage 20 in the dorsal side of host (CAG-GFP) at the same stage (fig. 4.11 C-D) and in the ventral side of the host as control transplants (fig. 4.11 A-B). When dorsal transplants were analysed at stage 37 cardiac induction was found (90% n=20) as indicated by the expression of GFP (fig. 4.11 C-D). Transplants were also analysed by double WMISH at stage 40 for the expression of MLC2 and MLC1v (fig. 4.11 G-G1). 35% of reaggregates transplanted in the dorsal region of the host (n=-84) showed regional gene expression distribution which included a region of overlapping expression between MLC2 and MLC1v and a region positive to MLC2 only (fig. 4.11 G1). Reaggregates co-injected with GATA-4 and Dkk-1 or injected with GATA-4 alone did not show any beating activity when transplanted in the dorsal side of the host embryos as in the case of reaggregates transplanted in the head of the host embryos (fig. 4.9). These regions in the host allow cardiac gene expression but are not permissive to the development of a secondary beating structure.

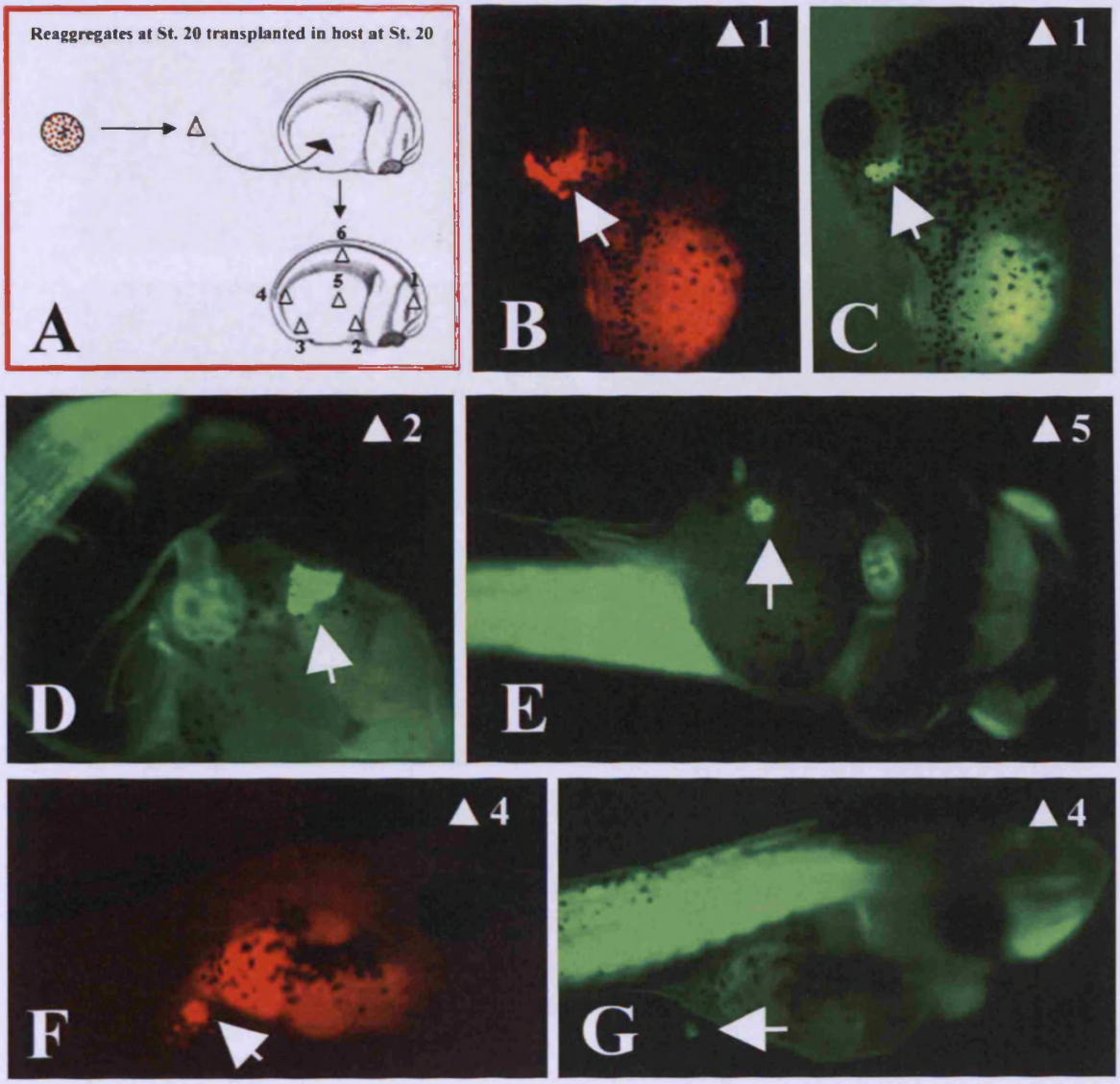

Figure 4.9 Secondary heart develops in a broad range of regions in the host. (A) Reaggregates at stage 20 were transplanted into host embryos at the same stage in a number of different regions rostrally, ventrally, laterally, caudally and dorsally, as indicated by the number. (B-C) 51% of reaggregates (CAG-GFP, n=20) (arrows) showed cardiac induction when transplanted into the head of the hosts but lost the ability to beat. (D-G) Reaggregates transplanted in the ventral region, lateral region or caudal region of the host were positive for GFP and showed beating activity (arrows).

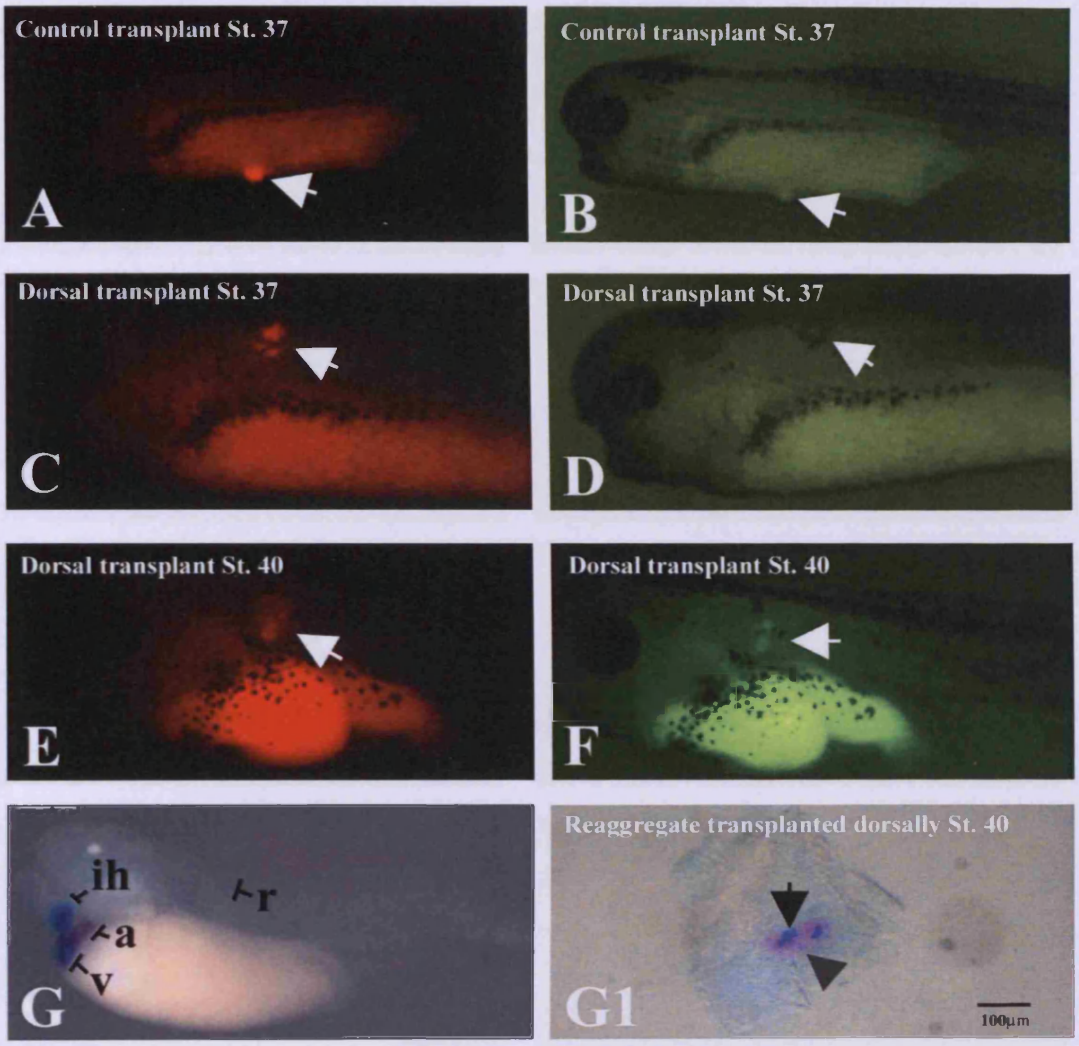

Figure 4.10 Delay in cardiac induction response in GATA-4 reaggregates transplanted close to the neural tube. (A-B) CAG-GFP reaggregates transplanted at stage 20 into hosts at the same stage (control transplant) were positive for GFP expression when analysed at stage 37 (arrows). (C-D) Reaggregates (CAG-GFP) were transplanted at stage 20 into the neural tube of hosts at the same stage (dorsal transplants). They did not show GFP activity (100% n=20) when analysed at stage 37 (arrows) but (E-F) showed cardiac gene expression when analysed at stage 40 (50% n=20; arrows). (G-G1) Gene expression was also confirmed by WMISH analysis for MLC2 and MLC1v expression. 20% of reaggregates (n=94) expressed MLC2 (head arrow) and only a small region of cells displayed an overlapping expression between MLC2 and MLC1v (arrow). Dorsal transplants did not show any beating activity. a; atria, ih; interhyoid muscles of the jaw; r; reaggregate, v; ventricle.

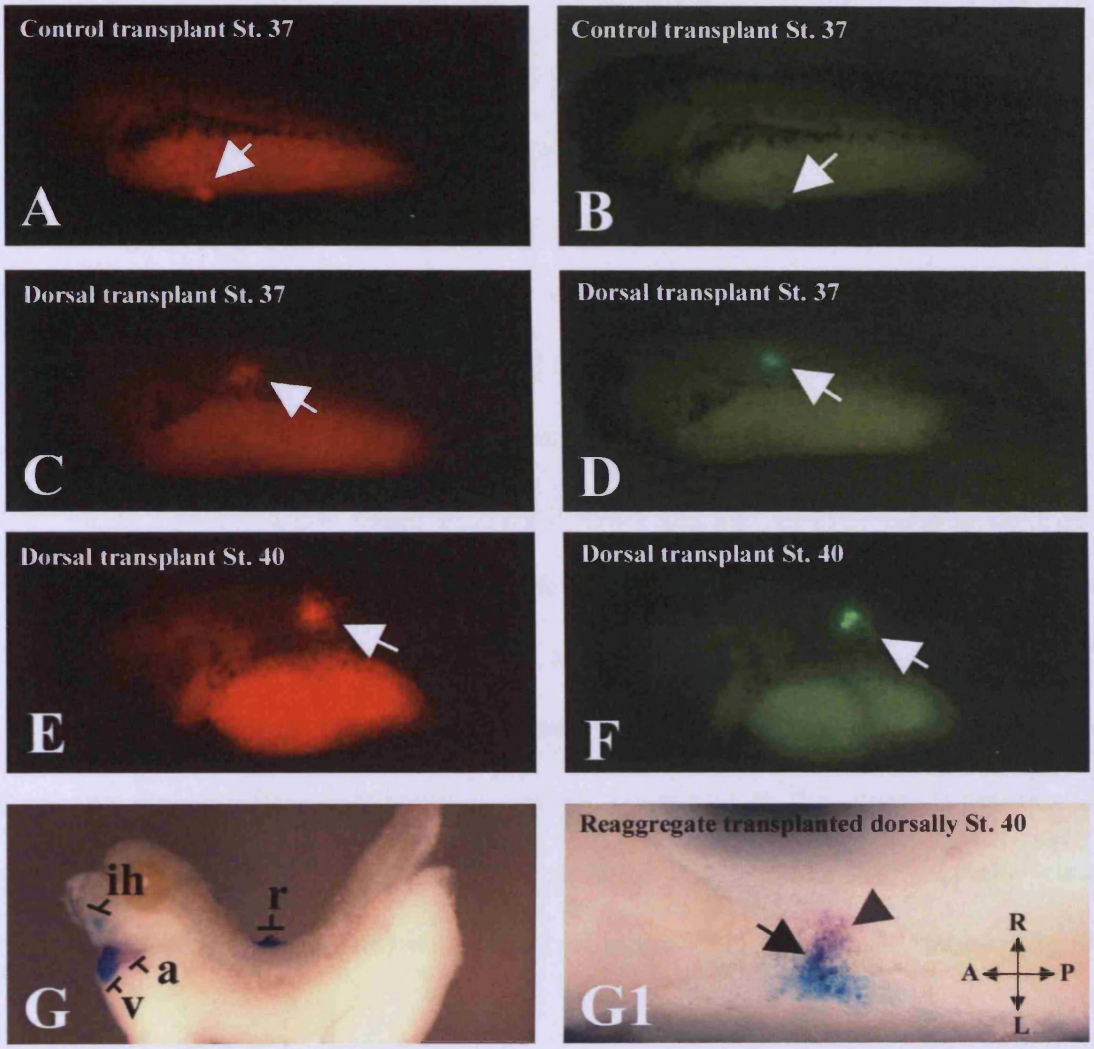

Figure 4.11 Co-injection with Dkk-1 prevents cardiac induction delay in neural tube transplant. (A-B) Reaggreagates (CAG-GFP) transplanted at stage 20 into hosts at the same stage showed cardiac gene expression when analysed at stage 37 (arrows) (C-D) Reaggregates (CAG-GFP) transplanted at stage 20 close into the neural tube in host at the same stage (dorsal transplants) showed GFP activity when analysed at stage 37 (90% n=20; arrows) and (E-F) when analysed at stage 40 (90% n=20; arrows). (G-G1) Gene expression was also confirmed by WMISH analysis for MLC2 and MLC1v expression. 35% of reaggregates (n=84) showed overlapping expression between MLC2 and MLC1v (arrow) and a region positive to MLC2 alone (head arrow). Dorsal transplants did not show any beating activity. a; atria, ih; interhyoid muscles of the jaw; r; reaggregate, v; ventricle. Anterior (A)-posterior (P) and left (L)-right (R) axes are indicated.

4.2.4 Competence to generate a beating secondary heart is lost by stage 28

Reaggregates at stage 20 showed the capacity to generate a SH when transplanted into host embryos at the same stage (46% of reaggregates showed beating activity n=220; fig. 4.1). We wanted to investigate if the reaggregates maintain the ability to generate a SH when transplanted at different developmental stage in host embryos at stage 20. For this reason a series of different transplants were performed using reaggregates at stage 13, 24, 26 and 28 (fig. 4.12 A). Reaggregates (CAG-GFP) transplanted at stage 13, 24 and 26 and analysed by fluorescent microscope at stage 40 were positive for GFP expression (fig. 4.12 C-E). The same set of transplants also showed beating activity (fig. 4.12 B). When reaggregates at stage 28 where transplanted into hosts at stage 20 and analysed at stage 40 they displayed weak cardiac induction and no beating activity (fig. 4.12 I-J). Moreover, those reaggregates had a different morphology when compared to the other transplants. The reaggregates had not developed a compacted structure but showed a layer of cells (some of them positive to cardiac markers) with clear cell migration into the host embryo (fig. 4.12 I-J).

Competence of reaggregates to generate a secondary heart occurs before stage 20 and is maintained until stage 26. The competence to generate a secondary structure in reaggregates transplanted at stage 24 and 26 is lower with respect to reaggregates transplanted at stage 13 and stage 20 (fig. 4.12 B). Therefore the competence is directly related with the developmental stage and is lost by stage 28 (fig. 4.12 B and I-J).

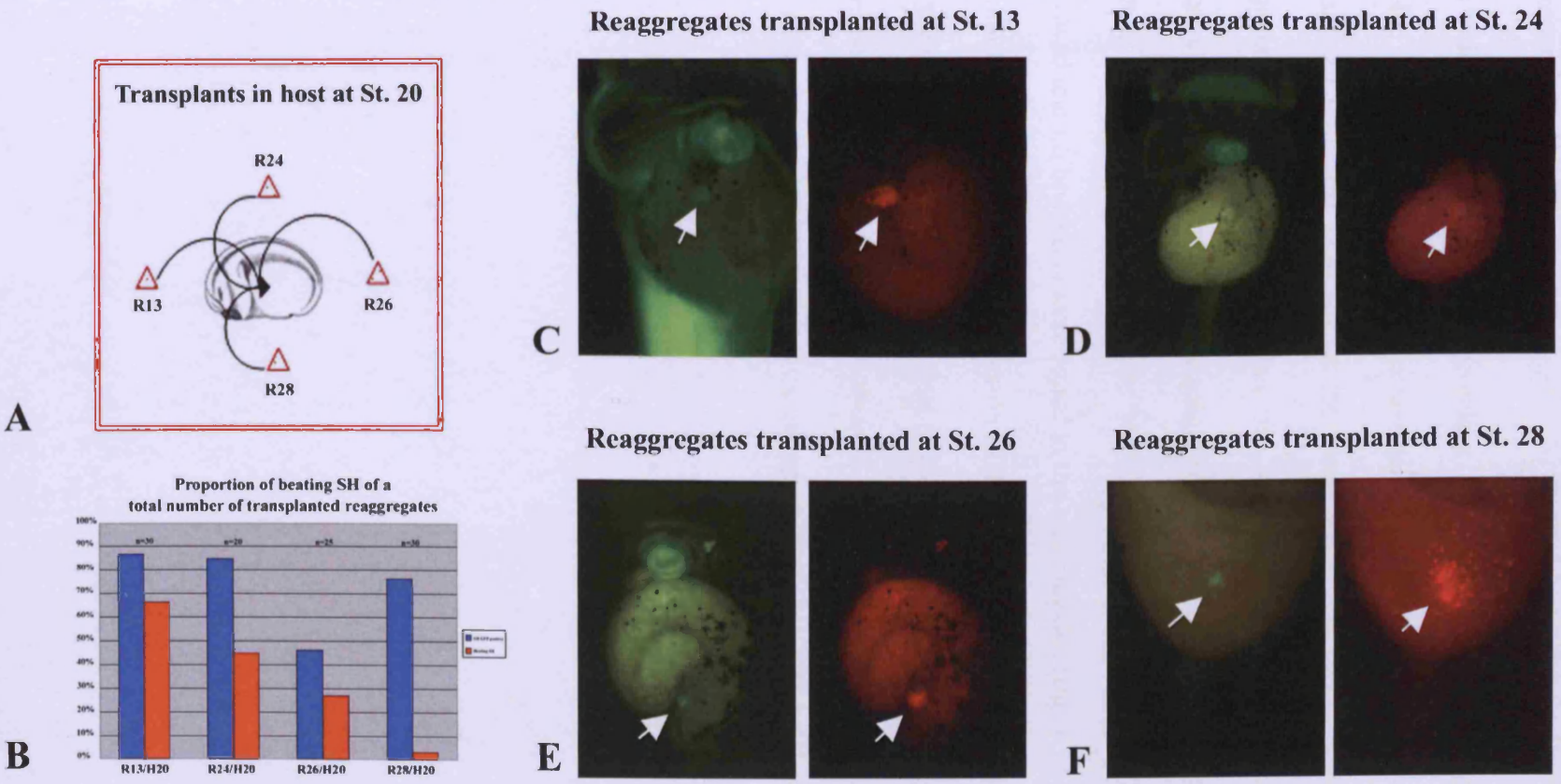

Figure 4.12 Competence to generate beating structure is maintained until St. 26 in reaggregates. (A) Reaggregates were transplanted at different developmental stages into host at stage 20. (C-E) Competence to generate a beating SH is maintained in reaggregates transplanted at stage13, 24 and 26 but is lost at stage 28. Reaggregates (CAG-GFP) were positive for GFP expression in all transplant experiments (arrows). (B) Summary of frenquency with which the SH were positive to the GFP expression and showed beating activity. Capacity to generate a beating structure declines with the increasing age of the reaggregates. R; reaggregate, H; host.

4.2.5 There is no cellular contribution from the host in the generation of the secondary heart

In order to understand if the secondary heart is generated exclusively from the transplanted cells, or, if the host has cellular contribution we transplanted GATA-4 injected reaggregates at stage 20 into host embryos at the same stage. The hosts were uniformly injected with Rhodamine-dextran lineage tracer (fig. 4.13 A). This experimental design allowed any red host cell to be detected in dark reaggregate under the fluorescent microscopy using a DSR filter. Transplanted host embryos were analysed at stage 40. The 2D analysis performed showed that no host cells were found in the reaggregates (fig. 4.13 B-E). However, we can not exclude that there is cell contribution of the host in the regions of the reaggregates which were not visible in the surface of the host (fig. 4.13 B1, C1, D1 and E1; n=20). No further analysis has been carried out in order to obtain confirmatory results but from this preliminary finding it seems that the host influences the reaggregate to generate a SH non-cell-autonomously.

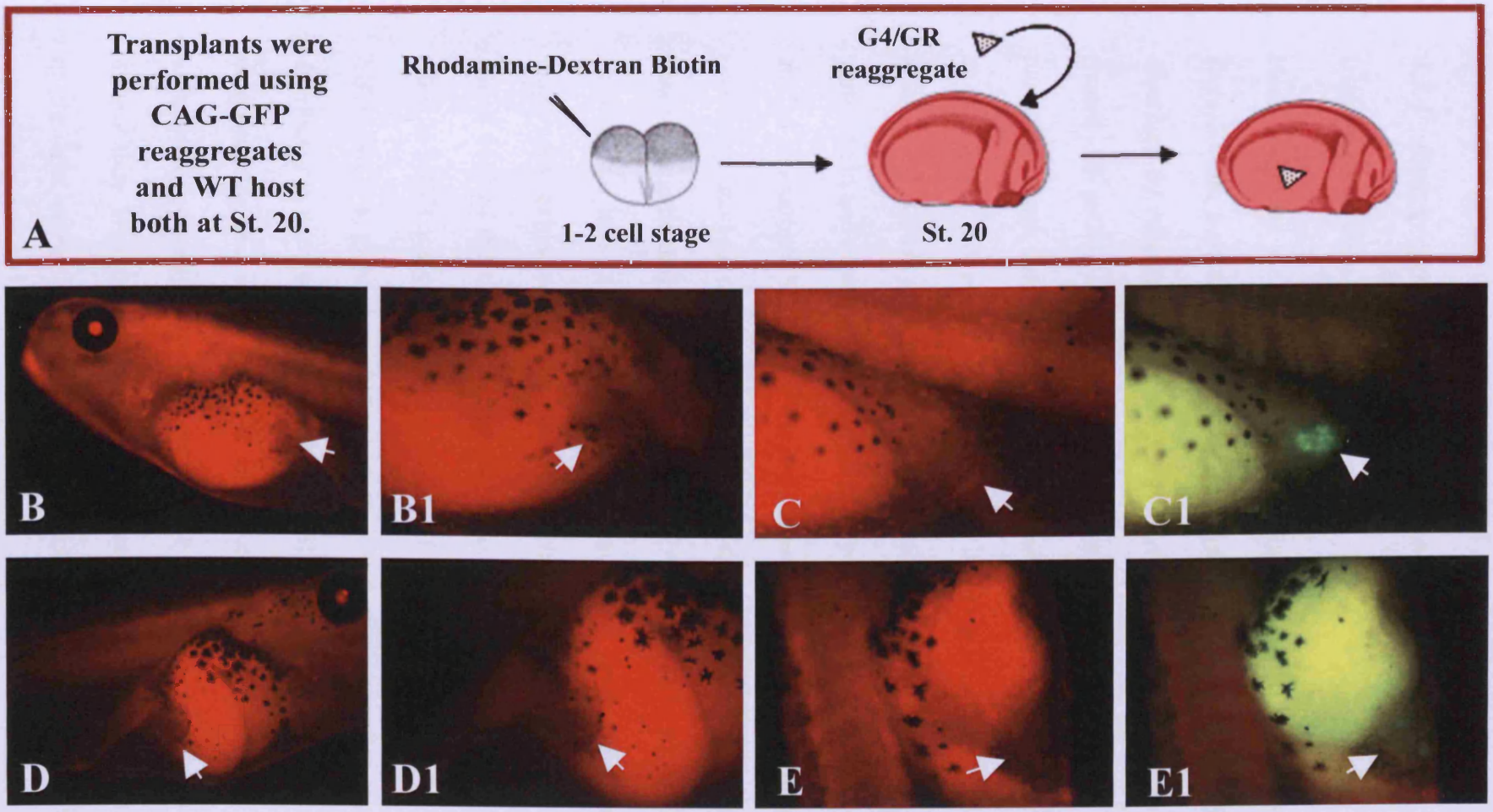

Figure 4.13 The reaggregate develops a secondary structure without any apparent cell contr bution from the host. (A) CAG reaggregates were transplanted into host embryos previously injected with lineage tracer at stage 20 and analysed at stage 40. (B-E) Reaggregates generate SH without host cells contribution (arrows).

4.3 Discussion

4.3.1 Reaggregate generates a secondary heart-like structure when transplanted into host embryo

Heart formation assay involves the transplantation of GATA-4 injected reaggregate at stage 20 into host embryo at the same stage. This assay is a modification of the assay described by Ariizumi and colleagues, in which dissociated and reaggregated cells were treated with activin and transplanted into host embryo at stage 20 (Ariizumi *et al.*, 2003). It has already been showed that activin is a mesoderm inducer (Asashima *et al.*, 1990; Green *et al.*, 1990; Smith *et al.*, 1990) and that its inducing activity is dose-dependent (Ariizumi *et al.*, 1991; Green *et al.*, 1992; Okabayashi and Asashima, 2003). Reaggregates treated with activin and transplanted into host embryos develop a beating structure with properties similar to a normal heart. Ariizumi and colleagues examined the histological section of the secondary heart showing that it can be divided into two chambers (atrium and ventricle) based on the thickness of the myocardium (Ariizumi *et al.*, 2003). In our experiments we showed that it is possible to generate a secondary heart (SH) using GATA-4 injected reaggregate explants. We have found that the use of GATA-4 generate a reliable assay in which 46% of the transplanted host embryos displayed beating SH (fig. 4.1). Moreover, using WMISH analysis for the expression of MLC2 and MLC1v we demonstrated that the SH showed a patterning of expression in which the cardiac markers are regionally distributed in the transplant (fig. 4.3). The SH also expressed proepicardial marker Tbx18 demonstrating that a further heart cell diversification has occurred (fig. 4.4). The gene expression patterning relative to the position of MLC1v and Tbx18 cells in reaggregates did not show alignment with the A-P expression patterning of the same genes in the heart of the host embryos. Thus, GATA-4-injected reaggregates transplanted into the host

embryos express pan-myocardial marker MLC2 and show a distinct cell diversification with the expression of myocardial and non-myocardial genes.

4.3.2 Host embryo is the key regulator of the SH development

Experiments performed using transplanted reaggregates demonstrated that host embryos are fundamental during the development of the SH. We showed that host embryos provide broad permissive regions, ventrolateral and caudal, where the beating SH can develop (fig. 4.9). When the reaggregates were transplanted in the head region of the hosts they were positive for cardiac actin gene expression but they did not show any beating activity. It has been suggested that GATA-4 induces the development of cardiac ectopic tissue in the head region without disruption of the heart development (Jiang and Evans, 1996). We believe that, as in the case of ectopic cardiac tissue development, the head region allows the reaggregates to express cardiac gene but does not support the development of beating structure (fig. 4.9). The systematic exploration of permissive regions into the host embryo directed us to transplant GATA-4 reaggregates into the dorsal region of the host. When GATA-4 reaggregates were analysed at stage 37 they showed a delay in expressing cardiac markers with respect to reaggregates transplanted in the ventral region of the host at the same developmental stage. The reaggregates transplanted in the dorsal region showed cardiac gene expression only later than stage 39 and did not show any beating activity. Moreover, WMISH analysis showed low degree of cardiac cell diversification. 20% of reaggregates showed a small number of cells with overlapping expression between MLC2 and MLC1v (fig. 4.10). We believe that the delay to respond to the inductive signal depends from the inhibitory activity of Wnt pathway. Dorsal region is regulated by the expression of Wnt signal which it has been shown to acting as repressor of cardiogenesis in

the chick neural tube (Tzahor and Lassar, 2001). In frogs, Samuel and Latinkic, showed that activation of Wnt/β-catenin pathway during gastrulation has no effect on cardiogenesis, while the same pathway blocks cardiogenesis during a period after cardiac specification and the onset of cardiac differentiation (Samuel and Latinkic, 2009). However, they did not examine cardiac gene expression after stage 34 in explants with elevated Wnt signalling. This evidence is comparable with our finding in which Wnt/β-catenin pathway suppresses the cardiac inducing activity of GATA-4 reaggregates transplanted at stage 20 into dorsal side of the host embryos. However, we found difficult to explain the cardiac gene expression showed in the reaggregates analysed at stage 40. Gessert and Kuhl reviewed that during the different steps of cardiogenesis exists a condition in which there are high or low levels of Wnt/β-catenin signalling. In particular, the cardiac terminal differentiation is regulated by low levels of Wnt/β-catenin signal and high level of non-canonical Wnt signal in which Wnt-11 is the prime candidate (Gessert and Kuhl, 2010). We believe that Wnt-11 may be one candidate which allows the cardiac gene expression in reaggregates transplanted in the dorsal side at stage 40. Concordant to our finding, it has been showed in *Xenopus* that Wnt11r, homolog to Wnt11, is highly expressed in the neural tube from stage 17 and is required for the neural crest cell migration (Matthews *et al.*, 2008). In order to verify if Wnt/β-catenin signal is involved in the regulation of cardiac diversification in reaggregates transplanted into the dorsal side of host embryos we decide to use the Wnt/β-catenin antagonist Dkk-1. It has been shown that Dkk-1 is capable to enhance cardiac induction in the noncardiogenic ventral marginal zone by inhibiting the Wnt signalling pathway, in particular Wnt3a and Wnt8 (Schneider and Mercola, 2001). During embryo development, Dkk-1 is expressed in the Spemann's organiser and cardiogenesis begins in the immediately adjacent mesoderm where there is a low level of Wnt-3a and Wnt-8 (Schneider and Mercola, 2001). For this reason, we

decided to co-inject reaggregates with GATA-4 and Dkk-1, and transplant them in the dorsal region of the host embryo. In contrast to the result found with GATA-4 reaggregates, co-injected reaggregates showed cardiac gene expression from stage 37 as in control transplants. Moreover, 35% of the reaggregates showed a pattern of gene expression in which MLC2 overlapped with the expression of MLC1v (fig 4.11). Thus, we believe that Dkk-1, as well as Wnt11 in GATA-4 reaggregates, may promote cardiac differentiation protecting the reaggregates from the inhibitory activity of Wnt pathway. The co-injected reaggregates transplanted in the dorsal region however, did not show any beating activity (fig. 4.11). These transplanted reaggregates act as the reaggregates transplanted in the head region of the host embryos. In both of the experiments reaggregates expressed cardiac markers but did not show beating activity. We believe that the signals involved in the growth and beating activity are regionally restricted and are not present in the dorsal side of the host embryo.

4.3.3 Embryonic axes of the host are important to the secondary heart development

WMISH analysis in the reaggregates transplanted into host embryos showed that gene expression patterning has been established via the regional expression of MLC2 and MLC1v or Tbx18. The regional expression of MLC2 and MLC1v or Tbx18 in reaggregates is not aligned with the A-P gene expression of the same gene in the heart of the host embryos. Despite this evidence we believe that the mechanism by which the host influences the SH development involves also the presence of embryonic axes. Transplants placed in ventralised UV-treated host express pan-myocardial marker MLC2 (fig. 4.6) but not ventricular marker MLC1v (fig. 4.7). MLC2 expression is directly related to the

injection of GATA-4 but cardiac cell diversification observed in the control transplants is dependent on the action of the host. Thus, reaggregates transplanted into ventralised UV-treated hosts act as GATA-4 reaggregates in isolation which express MLC2 but lack ventricular and proepicardial gene expression (fig. 3.4). The lack of cell diversification in the reaggregates is correlated with the lack of proper signalling from the host. The asymmetric structure of the heart develops under the influence of the three body axis (D-V, A-P and L-R) which confer the polarity to the heart structure (Brand, 2003). Thus, we believe that the embryonic axes are not directly instructive because the SH (transplanted into the ventral side of the host) showed a gene expression polarity independent from the A-P body axis, but provide signals that are permissive to the formation of the SH. Moreover, we believe that the host contribute also to the growth of the reaggregate. We compared the size of reaggregates transplanted into UV-treated hosts to the size of reaggregates transplanted in control hosts. The fluorescent microscopy analysis confirmed that reaggreates transplanted into UV-treated host was 40% smaller when compared to reaggregates transplanted into the control host (fig. 4.8). The microscopy analysis, however, requires further elucidation. The reaggregates were verified only in 2D analysis because they were not excised from the host embryos. Thus, we do not know the real dimensional structure of the reaggregates transplanted into the control and UV-host embryos. However, WMISH analysis confirmed the analysis performed using fluorescent microscopy. Reaggregate transplanted UV-treated host showed a small number of cell positive for MLC2 expression (fig. 4.7), while the control reaggregates showed in the host surface a wide number of cell positive for MLC2 and for the overlapping expression between MLC2 and MLC1v (fig. 4.7). This validates the hypothesis that UV-treated hosts do not support the cell diversification and growth of the reaggregates.

4.3.4 Capacity to generate a SH is lost by stage 28

The capacity of the reaggregates to develop a SH is extended after the end of gastrulation to the postneurula stages (fig. 4.12). However, it decreases at the beginning of the cardiac differentiation and it is lost just before the heart tube stage (fig. 4.12). Heart forming potency of the reaggregates to generate a SH is present until stage 26 and is completely lost at stage 28. The reaggregates transplanted at stage 28 did not beat when analysed at stage 40, and the structure was not as compact as reaggregates transplanted at different developmental stages with evidence of extensive cell migration into the host embryo (fig. 4.12 F). We believe that reaggregates have responded initially to the inductive signal of GATA-4 to express cardiac genes but with the increasing age they are not capable to respond to the signal provided by the hosts. Sater and Jacobson have shown that the heart field is composed at post neurula stage of lateral and ventral mesoderm (Sater and Jacobson, 1990a). However, they have shown that the lateral mesoderm lost the capacity to generate the heart at stage 28 in a process called heart field restriction. When they combined the lateral mesoderm at stage 28 to the anterior lateral endoderm at stage 20 they discovered that the anterior endoderm inducing activity was not sufficient to restore the heart forming potency in the lateral mesoderm. In contrast, the control performed with the lateral mesoderm at stage 20 and the anterior endoderm at the same stage formed a beating heart. Thus, the reaggregates transplanted at stage 28, as the lateral mesoderm, have lost the heart-forming potency even under the influence of a potent signal source as the host or the anterior endoderm respectively. For this reason we believe that the reaggregates have lost the heart forming capacity before the transplantation occurred. In the future it will be interesting to analyse if the capacity to generate the SH is also related with the age of the host. For this reason we aim to transplant reaggregates at stage 20 in host at different developmental stages.

5. *Requirement for signalling in the heart development after cardiac specification*

5.0 Requirement for signalling in the heart development after cardiac specification

5.1 Introduction

In previous work we described that GATA-4 injected reaggregates are capable of generating a secondary beating heart (SH) when transplanted into host embryos. The host plays an essential role in the SH development. We demonstrated that a wide range of permissive regions into the host allow the reaggregates to generate a SH. However, the host cells do not become a part of the SH (fig. 4.13) suggesting that the role of the host is to provide signals to allow the development of the SH. Since our results point to cell-cell communication between the host and the reaggregate as being very important in the SH development, we turned our attention to identification of the key signalling pathways that mediate these interactions. Little is known about the pathways involved in chamber specification and morphogenesis in *Xenopus*. For this reason we relied on the work performed in other vertebrate models and in particular zebrafish. It has been shown that FGF signalling is involved in the establishment of the size and the proportion of the zebrafish ventricle chamber (Marques *et al.*, 2008; Reifers *et al.*, 2000). Moreover, evidence in mouse and chick supports the idea that FGF is also involved in heart looping along the L-R axis (Boettger *et al.*, 1999; Meyers and Martin, 1999). Similarly, it has been shown that Nodal plays an important role in the L-R asymmetry establishment of the heart in zebrafish. In particular evidence in zebrafish shows an involvement of Nodal during the heart looping movements (Baker *et al.*, 2008). We have investigated the role of these signalling pathways in *Xenopus* embryos after cardiac specification and compared the results

obtained to the findings in other vertebrate models. These preliminary experiments are essential to understand the involvement of specific signal pathways during cardiac chamber formation. Moreover, they direct us to understand which signals are provided from the host to the reaggregates and how they are important in the development of the beating SH.

5.1.1 Chapter Aim

Using specific inhibitors of signalling pathways in *Xenopus* whole embryos, we aimed to:

1. Investigate the phenotypes obtained in the heart by inhibition of signalling pathways after cardiac specification.
2. Determine the time of action of these pathways during the main steps of cardiac morphogenesis.

5.2 Results

5.2.1 FGF signal is involved in heart looping and chamber growth during cardiac differentiation

To investigate the role of FGF, we treated *Xenopus* embryos with SU5402 after cardiac specification. SU5402 is a potent inhibitor of FGFR1 tyrosine kinase activity preventing the autophoshorylation of the intracellular domain, which occurs upon the binding of the receptor with FGF molecules. SU5402 is composed of an oxindole core which occupies the binding site of the ATP preventing the nucleotide binding (Mohammadi *et al.*, 1997).

Embryos were treated with SU5402 in three different time intervals starting from stage 20 (fig. 5.1 A). Embryos were then collected at stage 39-40 to be analysed by WMISH for the expression of MLC2 and MLC1v. Phenotypes obtained in the heart of treated embryos were classified as Δ1 and Δ2. 75% of the embryos treated from stage 22 to 30 (n= 20) showed completed looping and the ventricle partially located on the left side of the body. Of these, the jaw muscles fused in the midline (50%) or were partially fused (fig 5.1 D2 and D4, Δ1; table 5.1). 25% of the embryos showed the ventricle completely located on the left side of the body (fig. 5.1 D3, Δ2; table 5.1). 50% of the embryos treated from stage 22 to 39 (n=22) showed a small ventricle with respect to control embryos, a fusion of the jaw muscles and a visible short body trunk (fig. 5.1 E2-E3, Δ1; table 5.1). The rest of the embryos (50%) showed ventricle anteriorisation and fusion of the jaw muscles (fig. 5.1 E4, Δ2, table 5.1). In both treatments the hearts showed defects in looping. In the last treatment performed (stage 30-39, n=21) embryos displayed defects in the ventricle location. In particular, 50% of the hearts showed a partial looping and the ventricle located anteriorly to fuse with the jaw muscle (fig. 5.1 F3, Δ2; table

5.1). The rest of the embryos showed completed looping with visible ventricle anteriorisation and fusion with the jaw muscle (fig. 5.1 F2 and F4, Δ1; table 5.1).

These experiments were informative in highlighting the later roles of FGF in heart formation. Inhibition of FGF between stage 20 and 39 causes two phenotypes:

1. Reduction of the ventricle size (fig. 5.1 E2-E3 Δ1; table 5.1)
2. Looping defects and the ventricle located on the left side of the embryo (fig. 5.1 D3 Δ2; table 5.1).

These results focused our attention on a short range of developmental stages which include stages 20 to 26.

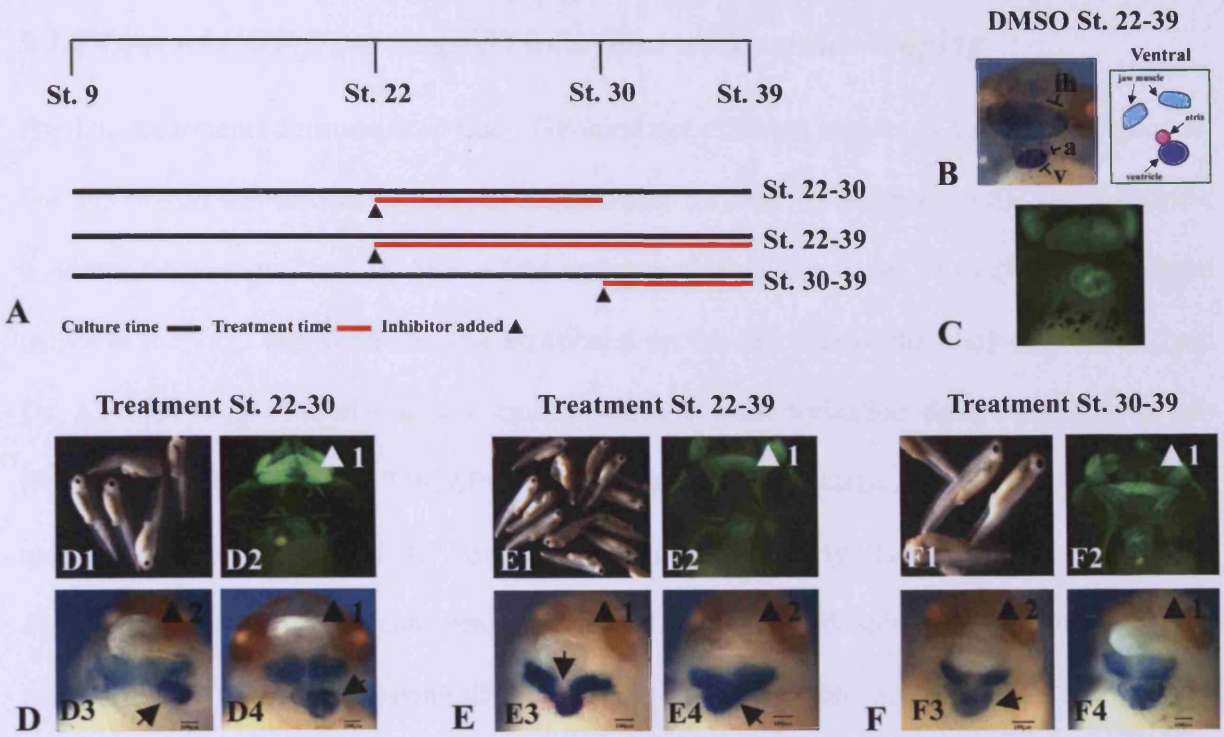

Figure 5.1 FGF pathway is involved in heart looping and chamber size growth. (A) Embryos were treated with SU5402 at different time periods and collected at stage 39. (B) Control embryos (CAG-GFP) were treated with DMSO from stage 22 to 39 and analysed by WMISH for the expression of MLC2 and MLC1v and (C) by the fluorescent microscopy. (D-F) Embryos treated with SU5402 were analysed at stage 40 using WMISH for the expression of MLC2 and MLC1v. (D) 75% of the embryos treated from stage 22 to 30 (n=20) showed correct looping (Δ1, arrow) while 25% of the embryos showed the ventricle located on the left side of the body (Δ2, arrow). (E) 50% of the embryos treated (n=22) from stage 22 to 39 showed a reduction in size of the ventricle (Δ1, arrow), while 50% of the embryos showed ventricle anteriorisation (Δ2, arrow). (F) 50% of the embryos treated from stage 30 to 39 (n=21) showed the ventricle fused with the jaw muscle and incorrect looping (Δ2, arrow). a; atria, ih; interhyoid muscle of the jaw, v; ventricle. St.; stage.

5.2.2 Loss of FGF from stage 23 interferes with cardiac looping

Previous treatments demonstrated that FGF regulates different aspects of heart morphogenesis. For this reason we refined the time of requirement for FGF by using short SU5402 treatment windows from stage 21 to 26. 36% of the embryos treated from stage 21 to 24 (n=38) showed incorrect looping. The ventricle was positioned on the left side of the body (fig. 5.2 D2 and D4, Δ2; table 5.1). 47% of embryos showed ventricle posteriorisation along the A-P body axis (fig. 5.2 D3, Δ1; table 5.1). 74% of the embryos treated from stage 21 to 26 (n=62) showed a unique phenotype in which the ventricle protruded posteriorly. The heart showed a strong defect in looping with a complete absence of a right-ward spiral movement. The OFT therefore, was repositioned ventrally (fig. 5.2 E1-E4, Δ1; table 5.1). 100% of the embryos treated from stage 23 to 26 (n=20) showed partial looping with the ventricle and the OFT lying perpendicularly to the A-P body axis (fig. 5.2 F1 and F3-F4, Δ 2; table 5.1). Moreover, 20% of these embryos displayed the ventricle located on the left side of the body (fig. 5.2 F2, Δ1; table 5.1). In both phenotypes the embryos had fusion of the jaw muscle in the ventral midline. 60% of the embryos treated from stage 24 to 26 (n=30) displayed defects in heart looping and small ventricle compared to embryos treated in DMSO (fig. 5.2 G1 and G3, Δ1; table 5.1). 40% of the embryos lacked of looping. The heart was located perpendicularly to the A-P body axis and the ventricle was smaller compared to control embryos. 30% of these embryos had the ventricle located on the left side of the body (fig. 5.2 G2 and G4, Δ2; table 5.1).

The experiment described showed that:

1. FGF likely regulates the looping of the heart and location of the ventricle along the A-P and L-R body axes (fig. 5.2 D-G; table 5.1).

2. FGF appears to regulate the ventricle chamber size (fig. 5.1 F, Δ1; fig. 5.2 G, Δ1; table 5.1).

These preliminary results suggest that FGF is important during the establishment of looping and possibly regulates the ventricle growth.

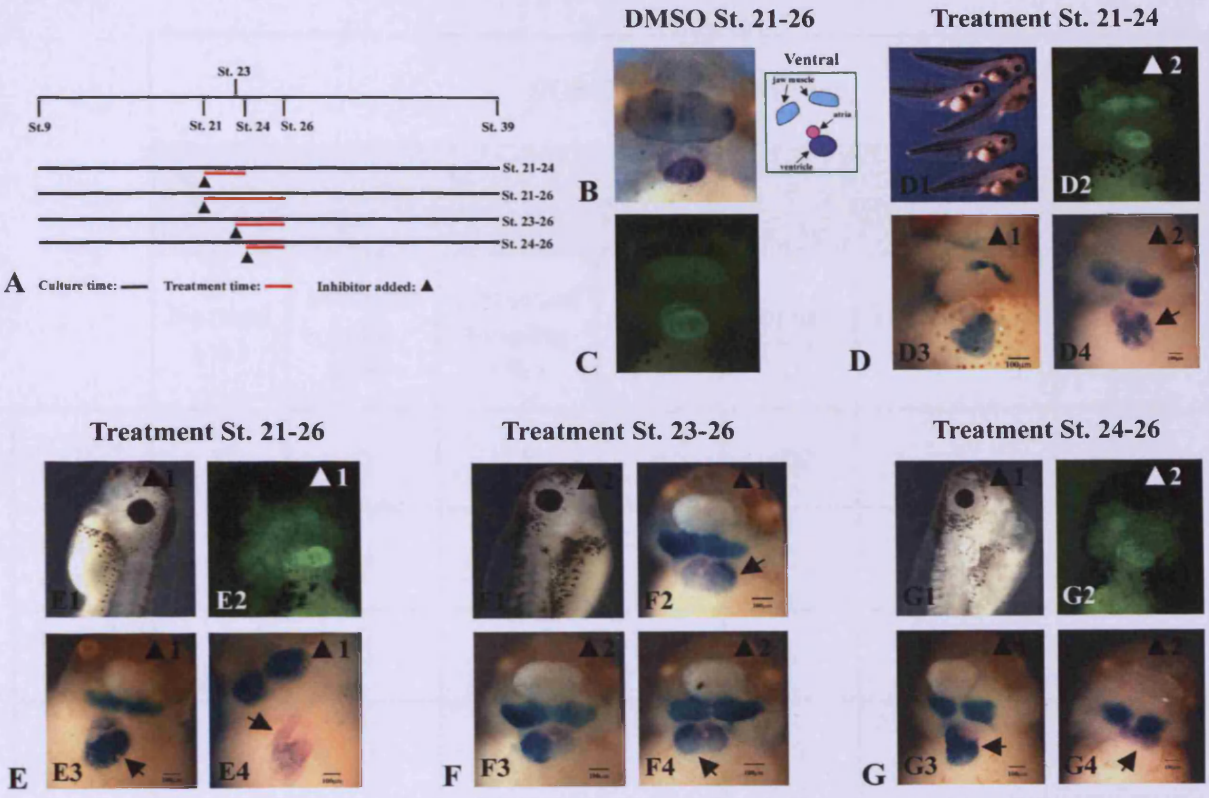

Figure 5.2 Inhibition of FGF interferes with heart looping and chamber development. (A) Embryos were treated with SU5402 at different time periods and collected at stage 40. (B) Control embryos (MLC1v-GFP) were treated with DMSO from stage 21 to 26 and analysed by WMISH for the expression of MLC2 and MLC1v and (C) by fluorescent microscopy. (D-G) SU5402 treated embryos (MLC1v-GFP) were analysed at stage 39 by fluorescent microscopy and by WMISH for the expression of MLC2 and MLC1v. (D) 36% of the embryos treated from stage 21 to 24 (n=38) had ventricle located on the left and incorrect looping (Δ2, arrow). (E) 74% of the embryos (n=62) treated from stage 21 to 26 showed OFT posteriorisation and lack of looping (Δ1, arrows). (F) 20% of the embryos (n=20) treated from stage 23 to 26 showed the ventricle located on the left side of the body (Δ1, arrow). The rest of the embryos showed the ventricle located on the right (Δ2, arrow). (G) 60% of the embryos (n=30) treated from stage 24 to 26 showed an unstructured heart with a low degree of looping (Δ1, arrow). 30% of the embryos had the ventricle located on the left (Δ2, arrow). a; atria, ih; interhyoid muscle of the jaw, v; ventricle, St.; stage.

	SU5402 TREATMENT						
	Looping phenotypes				Chamber phenotypes		
	Normal (%)	Straight tube (%)	Reversed looping (%)	Other (%)	Normal (%)	Distribution (%)	Size reduction (%)
St.22-30 (n=20)	75	0	25	0	100	0	0
St. 22-39 (n=22)	0	0	100	0	0	50	50
St. 30-39 (n=21)	50	0	0	50	0	100	0
St. 21-24 (n=38)	53	0	36	11	53	47	0
St. 21-26 (n=62)	26	0	0	74	26	74	0
St. 23-26 (n=20)	0	0	20	80	100	0	0
St. 24-26 (n=30)	0	0	30	70	0	0	100

Table 5.1 SU5402 treatments. Embryos treated with SU5402 showed defect in looping and chamber development. Looping phenotypes included embryos with normal looping, embryos with straight heart tube, embryos with ventricle positioned on the left side of the body (reversed looping) and embryos in which looping has occurred but was incomplete (other). Chamber phenotypes included embryos with normal chamber, irregular chamber position along the A-P body axis (distribution) and embryos with small chamber size with respect to control embryos. Each defect is showed in percentage of total number of embryos analysed.

5.2.3 A-83-01 treated embryos have defective heart looping

In order to investigate the role of Nodal during cardiac morphogenesis we used the soluble inhibitor A-83-01. A-83-01 is a potent inhibitor of Activin, TGFβ and Nodal pathways via the inhibition of the transcriptional activity of Alk4 (activin type IB receptor), Alk5 (TGFβ type I receptor) and Alk7 (nodal type I receptor) receptors (Tojo *et al.*, 2005).

Embryos were treated after stage 21 in order to compare the phenotypes obtained with embryos treated with SU5402. Embryos treated with A-83-01 from stage 19 to 22 (n=20) showed defects in looping. The heart tube was positioned perpendicular to the A-P body axis generating a semi circular shape (fig. 5.3 D, table 5.2). 70% of the embryos showed the ventricle located on the left side of the body (fig. 5.3 D2 and D4, Δ1; table 5.2). The rest of the embryos had the ventricle on the right (fig. 5.3 D3, Δ1; arrow). 50% of the embryos treated from stage 21 to 24 (n=40) lacked looping. The ventricles of these embryos were located on the right side of the body and the OFTs were more visible compared to control embryos (fig. 5.3 E1-E4, Δ1; table 5.2). Embryos treated from stage 21 to 26 (n=60) showed a randomisation of looping. 50% of the embryos had the ventricle positioned on the right side of the body (fig. 5.3 F1-F3, Δ1; table 5.2) while the rest of the embryos showed the ventricle located on the left (fig. 5.3 F4, Δ2; table 5.2). In embryos treated from stage 24 to 26 (n=32) two distinct morphological defects occurred (fig. 5.3 G; table 5.2). 30% of the embryos show a straight heart tube completely unlooped which lie along the A-P body axis (fig. 5.3 G1-G2 and G4, Δ1; table 5.2). The OFT and the ventricle were completely visible after WMISH analysis (fig. 5.3 G1-G2 and G4, Δ1; table 5.2). 50% of the embryos had ventricle located posteriorly and the distribution of the OFT and the atria is completely uncoordinated (fig. 5.3 G3, Δ2; table 5.2). As previously described A-83-01 inhibits TGFβ, activin and Nodal pathways. The

results found in A-83-01 treated embryos resemble the phenotypes described in embryos lacking of Nodal expression. However, we do not exclude that the defects in the heart showed in our results come also from the inhibition of TGFβ and/or activin. The phenotypes obtained in the embryos treated with A-83-01 showed:

1. Defect in L-R asymmetry establishment (fig. 5.3 D, Δ1)
2. Defects in heart looping (fig. 5.3 E-F and G3).

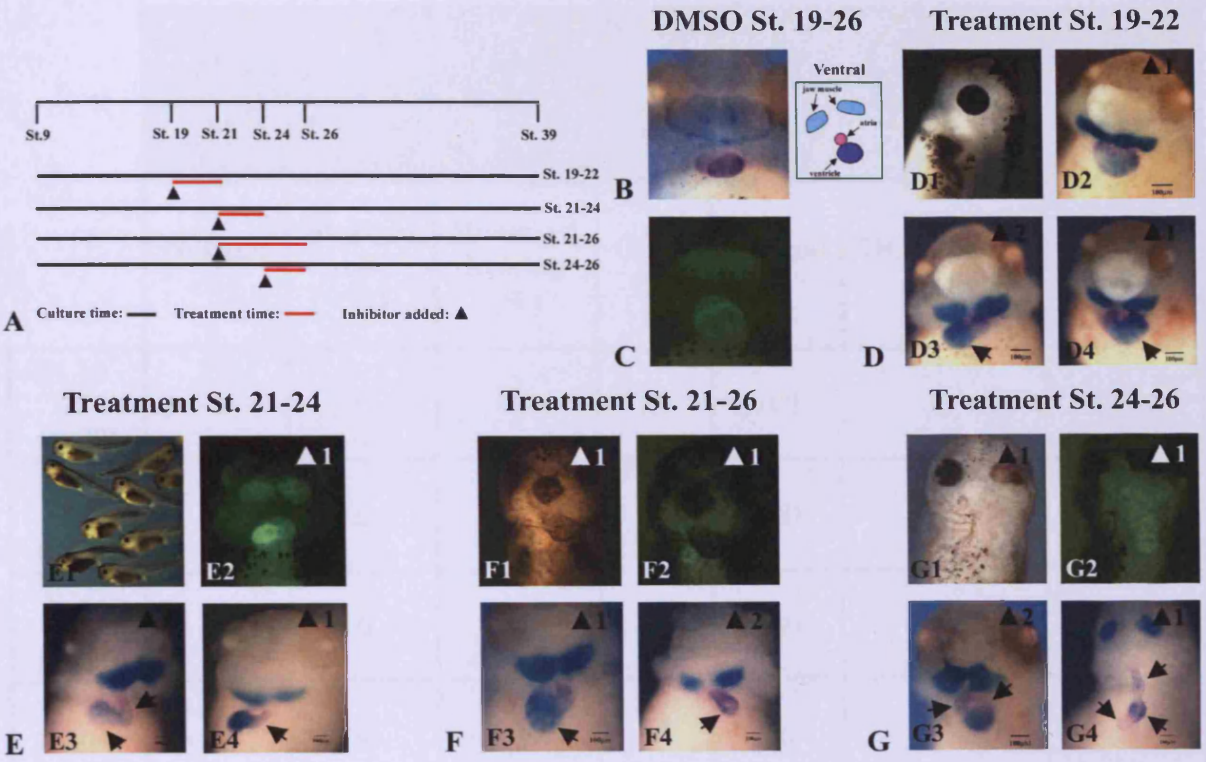

Figure 5.3 A-83-01 treated embryos showed defect in heart looping. (A) Embryos were treated with A-83-01 at different time periods and collected at stage 40. (B) Control embryos (MLC1v-GFP) were treated with DMSO from stage 21 to 26 and analysed by WMISH for the expression of MLC2 and MLC1v and (C) by fluorescent microscopy. (D-G) Embryos (MLC1v-GFP) were analysed at stage 39 by fluorescent microscopy and by WMISH for the expression of MLC2 and MLC1v. (D) 70% of the embryos treated from stage 19 to 22 (n= 20) showed the ventricle located on the left side of the body (Δ1, arrow). (E) 50% of the embryos treated from stage 21 to 24 (n=40) lacked looping of the heart and the ventricle was positioned on the right (Δ1, arrows). (F) 50% of the embryos treated from stage 21 to 26 (n=60) had the ventricle located on the right (Δ1, arrow) and 50% of the embryos had left-sided ventrcile (Δ2, arrow). (G) 30% of the embryos treated from stage 24 to 26 (n=32) lacked looping and the heart remained as a straight tube along the A-P axis (Δ1, arrows). 50% of the embryos showed uncoordinated looping and incorrect positioning of the OFT (Δ2, arrows). a; atria, ih; interhyoid muscle of the jaw, v; ventricle, St.; stage.

	A-83-01 TREATMENT						
	Looping phenotypes				Chamber phenotypes		
	Normal (%)	Straight tube (%)	Reversed looping (%)	Other (%)	Normal (%)	Distribution (%)	Size reduction (%)
St. 19-22 (n=20)	0	0	70	30	100	0	0
St. 21-24 (n=40)	50	0	0	50	100	0	0
St. 21-26 (n=60)	0	0	50	50	100	0	0
St. 24-26 (n=32)	20	30	0	50	50	50	0

Table 5.2 A-83-01 treatments. Embryos treated with A-83-01 showed defect in looping and chamber development. Looping phenotypes included embryos with normal looping, embryos with straight heart tube, embryos with ventricle positioned on the left side of the body (reversed looping) and embryos in which looping has occurred but was incomplete (other). Chamber phenotypes included embryos with normal chamber, irregular chamber distribution and embryos with small chamber size with respect to control embryos. Each defect is showed in percentage of total number of embryos analysed.

5.2.4 Comparison between SU5402 and A-83-01 treatment

Different group of embryos treated with SU5402 or A-83-01 showed similar phenotypes in the heart. In particular, SU5402 treatments performed at stage 21-24, stage 23-26 and stage 24-26 showed a common defect in the heart with embryos treated with A-83-01 at stage 19-22 and stage 21-26. The hearts displayed defects in looping movement and the ventricles were located on the left side of the body. A-83-01 treated embryos from stage 19 to 22 showed the highest percentage of left-sided ventricle (fig. 5.4). Thus, both treatments caused the same defect in heart looping. This work however, requires further investigation using alternative methods for manipulation of FGF and Nodal pathways.

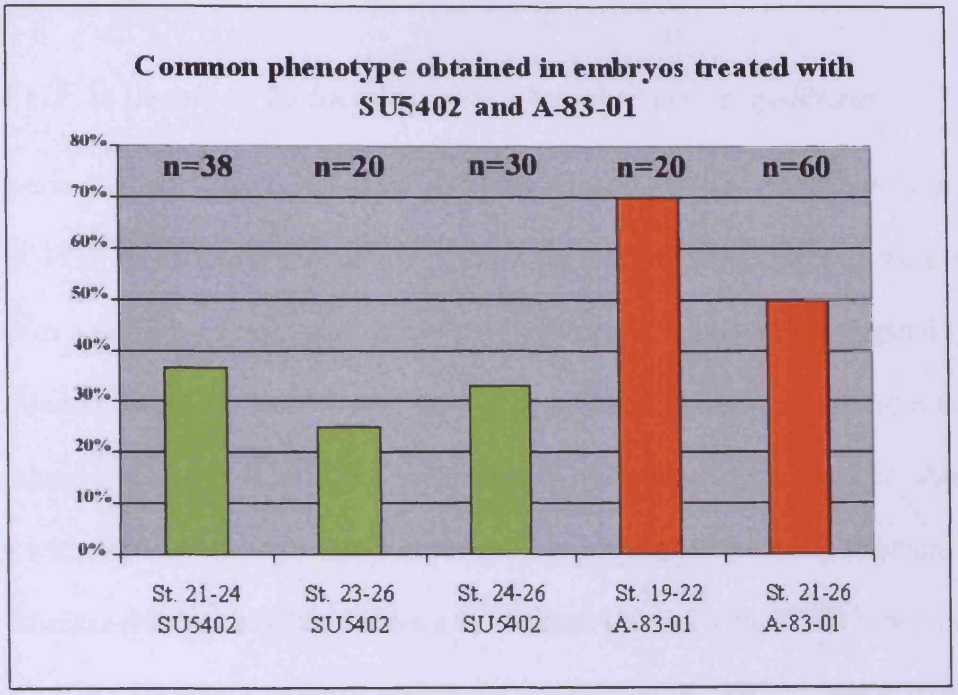

Figure 5.4 FGF and Nodal inhibitory treatments showed a common defect in the heart.

Summary of frenquency with which SU5402 treatments at stage 21-24, stage 23-26 and stage 24-26 showed a common defect in the heart with A-83-01 treated embryos at stage 19-22 and stage 21-26. The reversed looping caused the ventricle to be located on the left side of the body. The highest percentage of left sided ventricle was found in embryos treated with A-83-01 from stage 19 to 22. St; stage.

5.3 Discussion

5.3.1 FGF is involved in looping and chamber size regulation

The experiments performed in whole embryos using SU5402 (FGFR inhibitor) elucidated a role of FGF in different aspects of cardiac morphogenesis. Embryos were treated with SU5402 in a period between the end of cardiac specification and the beginning of cardiac differentiation. Previous work has shown in mouse and chicken a specific role for FGF in the regulation of the L-R axis. In particular, FGF8 mutant mice showed abnormal heart looping with 50% of the ventricle located on the left side of the body (Meyers and Martin, 1999). Similar results have been shown in chicken treated with FGF8 beads placed on the left side of the Hensen's node in which the expression of FGF is absent (Boettger *et al.*, 1999). In these embryos the heart looping resulted randomised and the 20% of the ventricle were located on the left. The usage of SU5402 in *Xenopus* embryos from stage 23 to 26 (fig. 5.2) showed similar results to mouse and chick embryos. The heart looping was reversed and 20% of the embryos show the ventricle located on the left side of the body. The heart, therefore, lacked the L-R axis and often lay perpendicularly to the A-P body axis. The reversed looping was also found in embryos treated from stage 21 to 24 and from stage 24 to 26 confirming that FGF regulated heart looping after cardiac specification and before the onset of cardiac differentiation. The embryos treated from stage 21-26, stage 24-26 and stage 21-24 (fig. 5.2) showed also defects in the OFT which was posteriorised if compared to control embryos. OFT anomalies were described in FGF8 mutant mice in which malpositioned or transposed aortic and pulmonary arteries arising from the right ventricle (outlet right ventricle, tetralogy of Fallot). The mutant mice showed also failure in the OFT septation or persistent truncus arteriosus (Abu-Issa *et al.*, 2002). Different evidence demonstrated that FGF is also important in the regulation of the ventricle

chamber development. These results were described in zebrafish where the *ace*/FGF8 mutants showed a small ventricle (Reifers *et al.*, 2000). Moreover, Marques and colleagues showed that FGF regulates the heart chamber development through the establishment of the correct number of cardiomyocytes from cardiac specification to heart tube formation. FGF inhibition reduced the number of cardomyocites in particular in the ventricle (Marques *et al.*, 2008). De Pater and colleagues showed that FGF is required for the addition of cardiomyocites in the arterial pole which are reduced in *fgf8*-MO injected zebrafish embryos. They suggested that the reduction of the ventricle size found in FGF mutant embryos (Reifers *et al.*, 2000) or in embryos where FGF was blocked (Marques *et al.*, 2008) is explained by the reduction of cardiomyocites differentiation in the arterial pole (de Pater *et al.*, 2009). In treated *Xenopus* embryos we found a reduction of the ventricle size in 50% of the embryos treated from stage 22 to 39 and also a small reduction in embryos treated from stage 24 to 26. The treatment from stage 22 to 39 covers a time period of 48 hours but the treatment performed from stage 24 to 26 covers a time period of few hours relative to the onset of the heart tube formation as showed in zebrafish. However, at present we do not know if the requirement for FGF in the regulation of the heart chamber size corresponds to the same found in zebrafish. For this reason, additional experiments need to be performed to confirm these initial results.

5.3.2 A-83-01 treated embryos showed defect in the L-R axis establishment in the heart

Xenopus whole embryos were treated with A-83-01 in the same time period as the embryos treated with FGFR inhibitor SU5402. A-83-01 is a potent inhibitor of ALK4, 5 and 7. Therefore, it inhibits TGFβ, actin and Nodal pathways. The phenotypic analysis performed in embryos treated with A-83-01 showed defects in the heart similar to that described in other model organism which lacked of Nodal expression. For this reason we believe that the effects obtained in embryos treated with A-83-01 were related to the lack of Nodal expression. However, we do not exclude that the same defects were caused by the inhibition of TGF β and activin pathways. We have found that embryos treated from stage 19 to 22 (fig. 5.3) generates a reversed looping in which 70% of the embryos showed the ventricle located on the left side of the body. The original position of the heart is altered so that the ventricle is located perpendicularly to the A-P body axis. Similarly 50% of the embryos treated from stage 21 to 26 showed a reversed looping but also defects in the OFT. We believe that the phenotype obtained in these embryos it may be related with the inhibition of Nodal which during early embryonic development is believed to initiate organ lateralization (Schier, 2003). Nodal is expressed in the LPM and activate the asymmetric expression of downstream gene *lefty2* (*lft2*). In the absent of Nodal signalling organ laterality is randomised or disrupted (Schier and Shen, 2000). The first organ to exhibit asymmetry in vertebrates is the heart (Ramsdell, 2005). In zebrafish it has been demonstrated that Nodal regulates directly the cardiac jogging via the asymmetric gene expression and indirectly the cardiac looping (Baker *et al.*, 2008). Charng and colleagues showed that a constitutive mutation of the Alk5 receptor leads to defects in heart looping and morphogenesis in mouse embryos. The unlooped hearts of these embryos, however, expressed cardiogenic factors such as MF20, dHAND and MLC2V. Thus, the constitutive

activation of Alk5 does not prevent cardiac myocyte differentiation (Charng *et al.*, 1998). This evidence demonstrates that either inhibition or overexpression of Alk5 generates defects in the heart looping and morphogenesis. A-83-01 treated embryos from stage 21 to 24 and from stage 21 to 26 (fig. 5.3) showed a posteriorised OFT which was misaligned with the ventricle. This type of phenotype has been extensively described in embryos lacking of the expression of the downstream Nodal target Pitx2. Inactivation of Pitx2 in the SHF of mice embryos showed severe defect in the artero-ventricular alignment of the OFT and in the proliferation of a subpopulation of the proximal OFT myocardium (Ai *et al.*, 2006). Comparison between the SU5402 and A-83-01 treated embryos (fig. 5.4) showed that both regulate the looping probably at different developmental stages. Embryos treated with SU5402 from stage 23 to 26, from stage 21 to 24 and from stage 24 to 26 showed left side embryos as well as in the embryos treated with A-83-01 from stage 19 to 22 and from stage 21 to 26 (fig. 5.4). We can not speculate if Nodal/activin/TGFβ regulates heart looping before FGF or if they interact at the same time. Moreover, we have not showed the effect of SU5402 and A-83-01 in embryos treated from stage 19 to 22 and from stage 23 to 26 respectively. In both of the treatments we did not obtained a clear data and for this reason they were not displayed together with the other treatments. Evidence showed that FGF and Nodal act differently in mouse and chick to regulate the L-R asymmetry. Meyers and Martin showed a schematic diagram summarising the interaction of Nodal and FGF8 during the embryo development in chick and mouse. In chick FGF8 is expressed in the right side of the Hensen's node and acts as right determinant inhibiting the expression of Nodal which is expressed in the left side. In contrast, in mouse FGF8 is required in the node for the left side expression of Nodal during the early phase and in the LPM during the late phase (Meyers and Martin, 1999). It is not known if in *Xenopus* FGF and Nodal collaborate in the establishment of L-R asymmetry during heart looping. Our preliminary

results require further investigation to clarify if the phenotypes described in A-83-01 treated embryos are related to the inhibition of Nodal or can also be attributed to the inhibition of TGFβ and/or activin.

6. *Discussion*

6.0 Discussion

In this study we have developed a novel model to investigate cardiac cell diversification and morphogenesis. The cardiogenesis assay has been used to study the regulation of cardiac cell diversification during the heart development. More complex events that constitute heart morphogenesis have been studied using the heart formation assay. In this assay we generated a secondary heart-like structure that showed growth, beating and rudimentary morphogenesis, all features of heart development that do not occur in cultured animal cap explants injected with GATA4. Compared to in vivo study of heart function, one advantage of the model is that the process of morphogenesis can be observed and precisely manipulated in a non-essential secondary heart. Together these assays provide new information concerning the mechanisms that regulate heart development.

6.1 Study of cardiac cell fate diversification in AC explants

Animal cap (AC) explants assay is a simple method to study the establishment of cardiac cell diversification through the injection of GATA-4 mRNA. AC explants analysed at stage 39 showed the expression of ventricular markers MLC1v and Irx4 and also the expression of pro-epicardial marker Tbx18 (fig. 3.1). The expression of these genes demonstrated that cardiac cell diversification has occurred in the explants which were composed of myocardial and non-myocardial tissue types. Moreover, using double-whole mount *in situ* hybridization in the same explants for the expression of MLC2 and MLC1v or cTnI and MLC1v (fig. 3.2) it has been found a gene expression patterning in which there were regions of overlapping expression between MLC2 or cTnI and MLC1v and regions in which there were no MLC1v expression. The manipulation of the explants in this assay has been useful to understand the mechanism involved in the establishment of the cardiac cell

diversification. Using dissociated and reaggregated GATA-4 injected explants we showed that the cardiac cell diversification was lost due to the lacking of MLC1v, Irx4 and Tbx18 gene expression (fig. 3.4). The dissociation and reaggregation in GATA-4 injected explants involved the lost of any cell-cell contact (Grunz and Tacke, 1989) and more importantly the lost of any embryonic pre-patterning established before the dissociation (Sokol and Melton, 1991). The expression of MLC2 in reaggregated explants is given by the injection of GATA-4 but the lack of any embryonic pre-patterning does not allow the cardiac diversification to happen. Using molecules known to be involved in the heart development as Dkk-1 we showed that is possible to rescue partially the expression of ventricular marker MLC1v (fig. 3.6). Dissociated and reaggregated explants previously co-injected with GATA-4 and Dkk-1 showed the expression of MLC1v when analysed at stage 39. However, despite the expression of MLC1v, the same explants did not show the expression of Irx4 and Tbx18 (fig. 3.6). Thus, we believe that the expression of MLC1v only in GATA-4 and Dkk-1 co-injected reaggregates is dependent entirely from the capacity of Dkk-1 to anteriorise the body structure during the embryo development (Glinka *et al.*, 1998). Similarly to Dkk-1 treated explants, we have found that the inhibition of BMP signalling through the co-injection of tBr mRNA, mutant form of the type I BMP specific receptor, with GATA-4 mRNA rescued the expression of MLC1v, Irx4 and Tbx18 in reaggregated explants (fig. 3.7). The usage of GATA-4 reaggregates or GATA-4 AC explants showed how is possible to study the cardiac cell diversification using a simple assay. In particular, co-injected reaggregates or GATA-4 reaggregates combined with Dkk-1 injected AC explants demonstrated that these explants can be easily manipulated and cultivated until required stage to detect the expression of specific genes. For this reason we believe that the usage of *Xenopus* ectodermal explants is a valid method to study the establishment of the cardiac cell diversification. We have shown that the ventricular and

proepicardial cell fate specification occurs after neurula stage. We achieved this conclusion through the use of dissociated and reaggregated heart field explants (fig. 3.5). These explants were cut from the embryos, dissociated and reaggregated at stage 20 and cultivated until stage 39. RT-PCR analysis showed that they express pan-myocardial marker MLC2 but not ventricular and proepicardial gene expression has been found (fig. 3.5 A). Using AC explants and heart field explants we showed that cardiac cell diversification occurs through the presence of embryonic pre-patterning, involves the expression of specific genes as GATA-4 (Latinkic *et al.*, 2003) and Dkk-1 (Schneider and Mercola, 2001) and probably occurs prior the beginning of heart differentiation. These results showed how the *in vitro* assay is suitable to study the mechanism of the heart organogenesis. The use of embryonic tissue that does not normally contribute to heart development was useful to understand how specific factors activate cardiac differentiation. Moreover, the same explants treated *in vitro* are not under the influences of the many cell-to-cell interaction and cell signalling events that occurs in the embryo during gastrulation (Latinkic *et al.*, 2003). Thus, in vitro *Xenopus* explants are suitable to study the mechanism involved in embryo organogenesis, the identification of many genes involved in the heart development and also it can be a valid method for advancing regenerative medicine (Asashima *et al.*, 2009). Despite the suitability of AC explants in vitro assay to study the cardiac cell diversification, we have to report that this method showed some limitations. AC explants and reaggregates explants did not survive in cultivation for more than five days. We could not, therefore, verify the expression of the atria specific marker ANF (Small and Krieg, 2000) which in *Xenopus* is expressed specifically in the atrium only at stage 49 (5 day pf). Moreover, we also wanted to study the different steps involced in the heart morphogenesis. Despite the capacity of GATA-4 injected AC explants to beat spontaneously in culture, we found that the same explants showed a low degree of

morphogenesis. GATA-4 injected reaggregates explants express the differentiation marker MLC2 but they never formed beating tissue in culture. Although, cardiogenesis assay is a simple method to study the heart patterning and cell diversification establishment but is not suitable to study heart morphogenesis. We required an assay which showed a high percentage of reproducibility and also the possibility to create a structure resembling the characteristic of an ordinary beating heart. In order to achieve that, we combined an *in vitro* and an *in vivo* assay to generate the heart formation assay.

6.2 Study of cardiac cells diversification and morphogenesis using GATA-4 reaggregates transplanted in host embryos

Studying the heart morphogenesis required the use of a different assay in which it was possible to generate a structure similar to the heart where the regional identity of different genes has occurred and the degree of morphogenesis was higher compared to AC explants. These requirements were met in the heart formation assay, which allows direct manipulation of all phases of cardiac patterning and morphogenesis in a non-essential embryonic structure. We used GATA-4 injected reaggregates at stage 20 and we transplanted them into host embryos at the same developmental stages. We found that transplanted embryos showed 46% of secondary beating structure when analysed at stage 39-40. The secondary structure resembled an ordinary heart. We found that the 34% of the secondary beating heart (n=43) showed morphology similar to the heart tube (fig. 4.5). Thus, this assay showed how an entire heart can be easily regenerated rather than study heart morphogenesis using AC explants in culture that rarely show cardiac functions. We also showed that differently from GATA-4 reaggregates, the cardiac cell diversification has occurred in the secondary heart which expressed ventricular and pro-epicardial specific genes (fig. 4.3; fig. 4.4). Thus, we believed that *Xenopus* embryo is a valid model to study heart organogenesis. The use of cell types such as embryonic stem (ES) cells and more recently induced pluripotent stem (iPS) cells to study the myocardial regeneration has attracted substantial interest from the scientific community (Kinoshita *et al.*, 2010). More importantly, the ability of the human embryonic stem cells (hESCs) to differentiate into contracting cardiomyocytes are anticipated to have a strong impact on how heart disease will be treated in the future (Vidarsson *et al.*, 2010). The human origin and the intact genome let the hESCs to be a suitable *in vitro* model to study the congenital heart malformations which affect about 1% of live births (Bruneau, 2008). However, despite the

strong potential in the future, stem cells derived cardiomyocytes still resemble simply aggregating group of cells which are functionally and structurally different from the adult cardiomyocytes (Vidarsson *et al.*, 2010). Even thought hESCs, IPs and ESC represent a valid method for future therapies in heart disease and heart regeneration we must underlie that results obtained from *in vitro* cardiomyocites differentiation are entirely based on the knowledge gained from developmental biology study through the use of model organisms as *Xenopus laevis*. It is believed that cardio-inductive signals, involved in the cardiomyocytes differentiation, originate from direct cell-cell contact and/or factors secreted from the adjacent endoderm (Vidarsson *et al.*, 2010). The future challenge in stem cells differentiation will be to design strategies that will allow the cells to reach higher degree of maturation *in vitro* (Vidarsson *et al.*, 2010). This capacity, however, has been already showed using induced *Xenopus* embryo explants from which an entire functioning structure has been developed (Ariizumi *et al.*, 2003; Kinoshita *et al.*, 2010). This assay clearly demonstrates how the use of the model organism *Xenopus laevis* can still contribute to study different aspects of heart development like heart morphogenesis. Kinoshita *et* colleagues showed that the secondary heart, previously transplanted into host embryo, resembled a structure similar to an ordinary heart (Kinoshita *et al.*, 2010). Physiological analysis of the secondary heart demonstrated that the cardiac contraction appeared to originate from the atrium toward the ventricle-like site. Moreover, the histological section showed that the secondary heart is composed of a ventricle-like region with a thick myocardium and a region atrium-like with a thin myocardium (Kinoshita *et al.*, 2010). We showed that the secondary heart, generated from GATA-4 injected reaggregated explants, expressed ventricular specific marker MLC1v and pro-epicardial marker Tbx18 (fig. 4.3; fig. 4.4). Moreover, these markers showed a patterning of expression. The ventricle cells or the pro-epicardial cells were located in the opposite pole of the atrium cells (fig. 4.3; fig.

4.4). Thus, the secondary heart showed a high degree of maturation, cardiac cell diversification and the physiological activity of beating. We also focused our attention on the existing interaction between the host embryo and the reaggregated explants in order to develop a secondary functional structure. The results obtained demonstrated that the host plays a significant role in the generation of the secondary structure. The reaggregates respond to the inductive signal released from the host until late developmental stages (fig. 4.12) and the host showed a wide range of permissive regions in which is possible to generate a secondary heart-like structure (fig. 4.9). GATA-4 injected reaggregates cultivated until late developmental stages did not express ventricular and pro-epicardial markers. However, if the same reaggregates were transplanted into host embryos they developed a secondary beating structure positive to ventricular and pro-epicardial gene expression. Transplanted reaggregates into host embryos, previously exposed to the UV-light, did not show any cardiac cell diversification and not beating activity has been found (fig. 4.7). Thus, the signals released from the host are instructive to let the reaggregate develops as a functional secondary heart-like structure.

6.3 Signalling in Xenopus heart development after cardiac specification

In the previous experiment we demonstrated that GATA-4 reaggregates transplanted into host embryos develop a secondary beating heart. The performed experiments elucidated that the host plays an important role in the generation of the SH. We believe this happens because the host provides signals that influence the SH development. Moreover, we showed that there is no cell contribution from the host to the reaggregate (fig. 4.13). In order to clarify the mechanism of cardiac morphogenesis, in the heart formation assay, we needed to study the signalling pathways which are known to be involved during the heart development. Little is known about the signals involved in the heart after cardiac specification and just some evidence arises from the *Xenopus* model. For this reason we tested pathways of signal in whole embryos, known to be involved in different aspects of cardiac morphogenesis in other model organisms. We used two drugs which are already known to inhibit pathway of signals involved in the different steps of heart morphogenesis like the chamber formation. We used FGF pathway inhibitory drug in whole *Xenopus* embryos after the cardiac specification and mainly we found that the inhibition of this pathway causes in the heart of the embryos looping defect, irregular ventricle position and reduction of the ventricle size (fig. 5.1; fig. 5.2; table 5.1). Similar effect has been found in the heart of the embryos treated with A-83-01 soluble drug. The inhibition of the activin/TGFβ/Nodal pathways causes, in the heart of the treated embryos, defect in the heart looping (fig. 5.3). We compared, then, the results obtained to what is known in other model organisms. We found that SU5402 treated embryos showed similar effect to FGF8 mutant zebrafish embryos which showed small ventricle (Reifers *et al.*, 2000). A-83-01 treated embryos showed in high percentages defect in the L-R axis establishment of the heart. Although A-83-01 inhibits TGFβ, activin and Nodal pathways we believed that the results obtained from treated embryos were related of the most with the inhibitory effects,

found in other model organism, of the Nodal pathway of signal (Baker *et al.*, 2008; Schier and Shen, 2000). The goal of these experiments was to verify the involvement of these signals during cardiac morphogenesis in *Xenopus* embryos and extend in future this knowledge in the transplant experiments to clarify the role of the host in the formation of a beating SH. However, before extending the use of inhibitory drugs in the transplants experiments, we have to gain, in the future, a wider knowledge on the effect of these drugs in all the steps of the heart morphogenesis.

6.4 Conclusion and future directions

The assays described in this thesis showed a simple system to study the cardiac diversification and the different aspects involved during the heart morphogenesis. Cardiogenic assay provide a simple *in vitro* assay using GATA-4 injected animal cap (AC) explants. These explants cultivated until late developmental stage showed the expression of ventricular and proepicardial markers and in rare cases were able to beat. This result demonstrated that the AC cells respond to the induction activity of GATA-4 and differentiated in myocardial and non-myocardial cell types. Moreover, the ventricular marker MLC1v showed patterning of expression when analysed using WMISH with MLC2. In contrast to the results found in AC explants, the ventricular and proepicardial gene expression was lost in dissociated and reaggregated AC explants. These explants expressed ventricular and proepicardial markers only when manipulated with Dkk-1 mRNA and BMPR inhibitor. In the future it would be interesting to further investigate the mechanism that leads cardiac cell diversification through the manipulation of GATA-4 injected reaggregates with molecules known to be involved during cardiogenesis. For example we want confirm the role of BMP in cardiac cell fate diversification using molecules as Dorsomorphin known to inhibit BMP type I receptor Alk2, -3 and -6 (Yu *et al.*, 2008). Adding Dorsomorphine to GATA-4 reaggregates at different developmental stage will clarify the time-specific involvement of BMP in establishing cardiac cell fate diversification. Similarly we want to manipulate GATA-4 reaggregates through the overexpression or inhibition of molecules as Retinoic acid known to be involved in the establishment of the anterior-posterior polarity in the heart (Hochgreb *et al.*, 2003).

When GATA-4 injected reaggregates were transplanted into hosts they developed a beating structure in the 46% of the cases demonstrating that the assay was stable and

reproducible. We showed that the development of the secondary heart (SH) is entirely dependent from the host activity. The host provides signals that allow the reaggregates to develop a beating structure. Little is known about the signals involved during the heart morphogenesis in particular in *Xenopus* model. For this reason we initiated a study of specific signalling pathways (known to be involved during cardiogenesis) in whole embryos during the heart morphogenesis. We provided for the first time evidences in *Xenopus* model for FGF and Nodal/TGFβ/activin involvement in heart looping and chamber growth. The treatments were performed in a time period between the end of cardiac specification and the onset of cardiac differentiation. The experiment performed in whole embryos aim to elucidate the role of these pathways in heart morphogenesis. We aim in the future to confirm the phenotype obtained using different inhibitors for the pathways studied, and extend our understanding though the manipulation of other signalling pathways known to be involved in the heart development. The results showed can be also extended in transplanted host embryos in order to verify which signalling pathways are involved in the development of the SH, by manipulating signalling in GATA-4 reaggregates in a period between the end of cardiac specification and the onset of differentiation. For example we aim to inhibit FGF pathways via the use of SU5402 (Mohammadi *et al.*, 1997), TGFβ/Nodal/activn pathways using A-83-01(Tojo *et al.*, 2005) and SB-431542 (Inman *et al.*, 2002), and verify the role of Wnt signalling with compounds such as Lithium Cloride known to activate the canonical Wnt pathway (Klein and Melton, 1996). The advantage of treating GATA-4 reaggregates with soluble compounds such as LiCl, SU5402 or A-83-01 is the ability to stimulate or inhibit signalling pathways transiently at different time points before transplantation into host embryos. This allows us to verify whether under specific treatments GATA-4 reaggregates have the capacities to generate a SH.

7.

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7.0 Bibliography

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