Chapter 6

The expression, regulation and function of *c125*, a target of Shh signalling, during zebrafish development

6.1: Preface

The following chapter describes the main aspects of the project that I carried out during the first two years of my PhD, which is unrelated to the rest of the project presented in this thesis. This initial project entitled 'The regulation of *c125* and its role in the zebrafish embryo' began successfully, but serious problems were later encountered. For the functional studies of *c125*, a morpholino-mediated knock-down approach was performed, which yielded a very interesting muscle phenotype (described later in this chapter). Having characterised this phenotype, I further assessed the effect of loss of *c125* during myogenesis, neurogenesis, and also the activation state of the Hh signalling pathway. It was during these investigations that I became aware that the initial muscle phenotype obtained by morpholino-mediated knock-down of *c125* was not reproducible. This is despite the fact that the morpholinos continue to down-regulate *c125* mRNA, as revealed by semi-Q PCR. It is unknown at which point in time that the morpholinos stopped producing the previously characterised phenotype, thereby making the subsequent *c125* loss-of-function studies unreliable. A great deal of time and effort was put into trying to re-establish the initial characterised muscle phenotype, but this was never achieved. As a result, all loss of *c125* function data became statistically non significant, and so I will not address the statistical significance of this data. In this chapter, I will present some of the data obtained until the point at which *c125* morpholinos were no longer found to have an effect.

6.2: Introduction

6.2.1: Expression of *C125* in the mouse embryo is regulated by Shh signalling

Clone125 (*C125*) was identified in a subtractive hybridisation screen which was carried out to identify transcriptional target genes of mouse Gli2 (thesis, Antonio Milano 2005). Confirming the fact that Hh signalling regulates the expression of *C125*, the progressive removal of *Shh* alleles in heterozygote or homozygote E9.5 mouse embryos causes a dose dependent reduction in *C125* expression. Similarly, a progressive loss of *Gli2* alleles also causes a progressive loss of *C125* expression throughout the embryo. However, weak *C125* expression still remains in the $Gli2^{-/-}$ embryo, although it is completely abolished in the double $Gli2^{-/-}/Gli3^{-/-}$ null embryo (thesis, Antonio Milano 2005). This suggests that Gli3 acts redundantly with Gli2 to activate *C125* in the mouse.

Accordingly, in situ hybridisation reveals that *C125* is expressed in sites of Hh signalling activity, in the E9.5 mouse embryo. *C125* is expressed in the ventral neural tube overlapping with the expression profile of Gli2, in addition to the eye, brain, myotome, gut, and branchial arches. Expression is also observed in the limb bud by E10.5 (thesis, Antonio Milano 2005). Overall, this expression pattern is conserved in the chick (unpublished data, Mark Watson 2007).

However, *C125* is mainly expressed as an anterior to posterior gradient in the mouse embryo (thesis, Antonio Milano 2005) despite *Shh* expression along the entire antero-posterior axis. This indicates that other regulatory pathways may be controlling the expression of *C125*. In agreement with this, *Shh* over-expression is not sufficient, but *Shh* is necessary, for *C125* activation (thesis, Antonio Milano 2005). It is possible that the opposing gradients of retinoic acid (RA) and fibroblast growth factor (FGF) along the antero-posterior axis, which have a role in ventral neural tube patterning (Diez del Corral *et al.* 2003), control *C125* expression. In this way, RA and FGF

could operate in conjunction with Shh and BMP. RA expressed anteriorly could induce *C125* expression in these regions, whilst it is possible that FGF inhibits *C125* posteriorly. Meanwhile, Shh induction of *C125* ventrally could be antagonised by dorsal BMPs (Briscoe and Ericson 2001). It is not known how C125 protein functions or precisely where it is expressed within a cell.

6.2.2: Chick *C125* plays a role in motor neuron differentiation

In the ventral neural tube, five distinct progenitor cell domains are generated at specific Shh concentration thresholds (*Figure 6.1*), which operate at sequential periods of time (Ericson *et al.* 1996). Initially, Shh signalling is needed to specify ventral progenitor cells, and is then required later for motor neuron differentiation (Ericson *et al.* 1996).

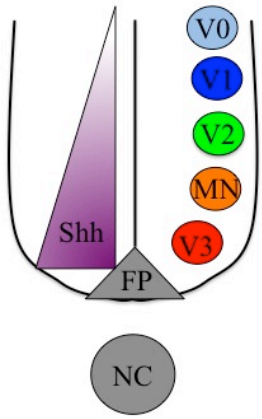

Figure 6.1: *A schematic representation of the ventral neural tube*. Five distinct neuronal progenitor domains (V3, motor neuron (MN), V2, V1 and V0) are generated in the ventral neural tube, in response to graded Shh signalling. The concentration of Shh is greatest at its source, the floor plate (FP). Shh is also secreted from the notochord (NC).

Shh is believed to regulate the expression of homeodomain and bHLH proteins (Jacob and Briscoe 2003), through the ratio of Gli activator to repressor forms, which decreases with increasing distance from the floor plate (Wang *et al.* 2000). Class I proteins are repressed by Shh signalling, whereas class II proteins are induced by Shh signalling (Briscoe *et al.* 2000). Different combinations of homeodomain proteins specify different domains, and cross-repressive interactions between the two classes of homeodomain proteins (class I and class II) in adjacent domains maintain each domain boundary (Briscoe *et al.* 2000).

The specific profile of homeodomain proteins controls the specific type of neuron formed. Motor neuron progenitor cells form in the domain comprising Nkx6.1 and Pax6 homeodomain transcription factor expression (Briscoe et al. 2000; Briscoe and Ericson 2001). *Nkx6.1* expression within the motor neuron domain allows for the bHLH protein *Olig2* to be expressed (Park *et al.* 2002; Novitch *et al.* 2003). This is the determination step in motor neuron formation, and *Olig2* expression causes an increase in *Ngn2* (bHLH) expression. Ngn2 drives the expression of *MNR2*, which is first expressed in the final division cycle of motor neuron progenitor cells (Jessell 2000;

Novitch et al. 2003), and acts to induce downstream factors such as Lim3, Islet1, Islet2 and HB9. These are post mitotic markers of motor neuron differentiation (Briscoe *et al.* 2000; Jessell 2000). It is thought that *C125* is a mediator of Shh signalling because its over-expression in the chick neural tube represses the class I protein Pax6 in the dorsal neural tube (unpublished data, Mark Watson 2007). However, there is also a slight ventral shift in the expression of Pax6, at the expense of the Nkx2.*2* positive V3 progenitor domain and the floor plate (*Figure 6.2*). This suggests an expansion of the motor neuron domain confirmed by observation of early up-regulation of MNR2 expression. Accordingly, an increased pool of somatic motor neurons, marked by premature Islet1 expression is also observed following *C125* over-expression. Results suggest that *C125* over-expression may cause precocious determination and maturation of motor neurons in the ventral neural tube.

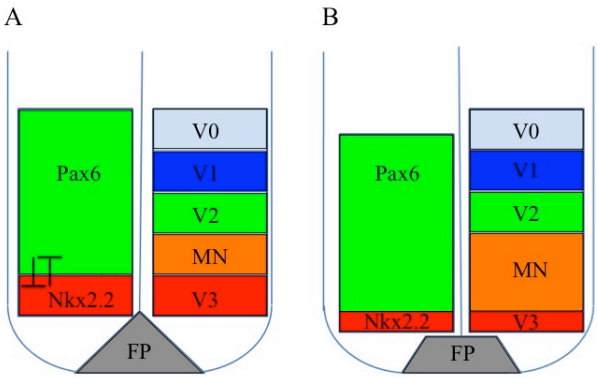

Figure 6.2*: A schematic representation of the effects observed following over-expression of C125 in the chick neural tube*. A: normal progenitor domain sizes and positioning in a wild-type neural tube. Repressive interactions between homeodomain proteins define distinctive neuronal progenitor domains. B: *C125* over-expression leads to a ventral expansion of Pax6, and a ventral expansion of the motor neuron domain, at the expense of the V3 domain and the floor plate (FP).

Loss of *C125* function by RNAi mediated knock-down in the chick neural tube does not cause the opposite effect of *C125* over-expression. Despite ventral contraction of Pax6 and Pax3 expression, and a slight dorsal expansion of Nkx6.1, no obvious effects are observed with the domains of expression of Olig2 or MNR2 (unpublished data, Watson 2007). This could be a result of only partial knock-down of *C125*. However, loss of *C125* does cause a reduction in Islet1 and Lim3 expression (unpublished data, Mark Watson 2007). Taken together, *C125* appears to regulate the determination of cells within the MN domain and also those within the V2 and V1 domains (which also express Pax3), but despite the disturbances to neuronal determination, cells within the motor neuron domain still acquire a normal fate. It is hypothesised that *C125* regulates specific motor neuron fate (unpublished data, Mark Watson 2007).

The alterations to the progenitor cell domains of the neural tube associated with over-expression or loss-of-function of *C125* are usually associated with changes in the concentration of Shh. For example, over-expression of *Gli2* causes a ventral contraction of Pax6 expression in the dorsal neural tube (Briscoe *et al.* 2000), an effect also caused by loss of *C125*. Over-expression of both

Gli2 and *C125* also causes up-regulation of MNR2 and Islet1 expression (Briscoe *et al.* 2000). *C125* therefore phenocopies *Gli2*. As a result, it is also hypothesised that *C125* has a role in interpreting the Shh gradient (unpublished data, Mark Watson, 2007). However, these studies lacked a knock-out model, and so the global effect of complete C125 knock-down was not observed.

6.2.3: The regulation of *c125* and its role in the zebrafish embryo

The conserved expression pattern of *C125* in the chicken and the mouse indicates that *C125* may be conserved throughout vertebrates. This also raises the possibility that *C125* is regulated by conserved mechanisms, and has a conserved function.

Therefore, I predicted that *c125* would be expressed in the zebrafish within the ventral neural tube, anterior CNS, eye, branchial arches, muscle of the somites, and the pectoral fins. I also predicted that expression of *c125* in each of these tissues would be regulated by Hh, and in the absence of Hh signalling a loss of *c125* expression would occur. To test these hypotheses, I searched for a *c125* ortholog in the zebrafish using bioinformatic software, and examined its expression pattern using in situ hybridisation techniques. To assess the role of Hh signalling in the regulation of *c125* in the zebrafish, I examined the *c125* expression pattern in zebrafish embryos that carry mutations in the Hh signalling pathway, such as *yot*, *smu*, and *ptc1/2* zebrafish. I also manipulated the Hh signalling pathway using cyclopamine and dnPKA mRNA.

I also hypothesised that *c125* would play a role in the formation of primary motor neurons in the zebrafish. I predicted that over-expression of *c125* mRNA would cause an expansion of *olig2.2* and *islet1* expression in the ventral neural tube, and that loss of *c125* function would cause a reduction in the expression of *islet1*. To test these hypotheses, I performed knock-down studies using a morpholino-mediated approach. This allowed the global knock-down of *c125* throughout the zebrafish embryo from the one-cell stage, and thus had the potential to reveal other important functions for *c125*. Likewise, injection of *c125* mRNA into the one-cell stage embryo could test the effect of *c125* over-expression throughout the embryo.

Finally, I tested the possibility that *c125* functioned within the Shh pathway, affecting the Shh concentration gradient. The analysis of the Hh-target gene *ptc1* in embryos over-expressing *c125* mRNA or embryos that lack *c125* as a result of morpholino-mediated knock-down would provide an account of the Shh gradient in the embryo.