Understanding Dlx-mediated effects on cell proliferation

by

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**ABSTRACT**

UNDERSTANDING DLX-MEDIATED EFFECTS ON CELL PROLIFERATION

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University of Guelph, 2014

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*Dlx5* and *Dlx6* are members of the *Distal-less homeobox (Dlx)* gene family and encode transcription factors essential for craniofacial patterning and differentiation of mineralizing cell types in the skeleton during development. Forced expression of *Dlx5* or *Dlx6* in progenitor cells promotes their differentiation and suppresses proliferation *in vitro* and *in vivo*. Conversely, studies involving cancer cell lines have shown that Dlx5 can promote proliferation, perhaps via direct transcriptional activation of cell cycle regulatory genes like *c-Myc*. We have shown that Dlx5 and Dlx6 inhibit cell growth in multipotent progenitor cells and primary limb bud cells and modestly upregulate *c-Myc* reporter transcription in such cells. Cumulative EdU-incorporation assays indicate that Dlx5 and Dlx6 decrease the proportion of actively cycling cells in a population and may increase the length of the cell cycle. Flow cytometry results suggest a higher proportion of cells in G1 compared to controls.
ACKNOWLEDGEMENTS

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Lastly, I would like to extend my gratitude to my friends and family for supporting me unconditionally throughout my university career. Without them, the completion of this degree would not have been possible.
DECLARATION OF WORK PERFORMED

All of the work in this thesis was performed by Rachel K MacKenzie.
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<th>Description</th>
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<tbody>
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<td>Ac-DEVD-AMC</td>
<td>N-acytly-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>AER</td>
<td>Apical ectodermal Ridge</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BP1</td>
<td>β protein 1</td>
</tr>
<tr>
<td>BRDU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone morphogenic protein 2</td>
</tr>
<tr>
<td>C terminal</td>
<td>Carboxy terminal</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>Cdx</td>
<td>Caudal-type homeobox</td>
</tr>
<tr>
<td>CEF</td>
<td>Chick embryonic fibroblast</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin dependent kinase inhibitors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CNC</td>
<td>Cranial neural crest</td>
</tr>
<tr>
<td>CycE</td>
<td>Cyclin E</td>
</tr>
<tr>
<td>dDREF</td>
<td><em>Drosophila</em> DNA replication-related element binding factor</td>
</tr>
<tr>
<td>hDREF</td>
<td>Human DNA replication-related element binding factor</td>
</tr>
<tr>
<td>Dll</td>
<td>Distal-less</td>
</tr>
<tr>
<td>Dlx</td>
<td>Distal-less homeobox</td>
</tr>
<tr>
<td>Dlx1</td>
<td>Vertebrate distal-less homeobox 1</td>
</tr>
<tr>
<td>Dlx2</td>
<td>Vertebrate distal-less homeobox 2</td>
</tr>
<tr>
<td>Dlx3</td>
<td>Vertebrate distal-less homeobox 3</td>
</tr>
<tr>
<td>Dlx4</td>
<td>Vertebrate distal-less homeobox 4</td>
</tr>
<tr>
<td>Dlx5</td>
<td>Vertebrate distal-less homeobox 5</td>
</tr>
<tr>
<td>Dlx5HD(^M)</td>
<td>Mutated Dlx5 with 3 alanine substitutions in the homeodomain</td>
</tr>
<tr>
<td>Dlx6</td>
<td>Vertebrate distal-less homeobox 6</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>dpt</td>
<td>Days post transfection</td>
</tr>
<tr>
<td>DRE</td>
<td>DNA replication-related element</td>
</tr>
</tbody>
</table>
DTT  Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
EdU  5-ethyl-2’-deoxyuridine
FITC  Fluorescein isothiocyanate
G0  Resting phase of cell cycle
G1  Gap 1 phase of cell cycle
G2  Gap 2 phase of cell cycle
G418  Geneticin
GF  Growth fraction
GFP  Green fluorescent protein
GMC  Ganglion mother cell
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOXA10  Homeobox A10
IRS-2  Insulin receptor substrate-2
L  Ladder
LI  Labeling index
M  Mitosis phase of cell cycle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msx</td>
<td>Msh homeobox</td>
</tr>
<tr>
<td>N terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Osc</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>PA1</td>
<td>First pharyngeal arch</td>
</tr>
<tr>
<td>PA2</td>
<td>Second pharyngeal arch</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenemine</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLB</td>
<td>Passive lysis buffer</td>
</tr>
<tr>
<td>Pros</td>
<td>Prospero</td>
</tr>
<tr>
<td>Prox1</td>
<td>Prospero related homeobox 1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>S</td>
<td>DNA Synthesis phase of cell cycle</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Tc</td>
<td>Cell cycle time</td>
</tr>
<tr>
<td>Tcrβ</td>
<td>T-cell receptor β</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>VP16</td>
<td>HSV-1 Viral protein 16</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Proliferation and Differentiation during development

Embryonic development is a complex process that involves the strictly controlled and highly co-operative interaction of a large network of genes involved in cell growth and proliferation, death and differentiation, metabolism, migration and communication (Barolo & Posakony 2002). Changes in gene expression during differentiation cause a change in cellular phenotype, behaviour and functional competence. This includes a loss of the ability to divide, mediated through the downregulation of genes involved in progression through the cell cycle and/or upregulation of genes inhibiting this progression. Proliferation and differentiation have long been understood to be incompatible cellular processes, and exit from the cell cycle is a hallmark of differentiation in most cell types, as is reflected in the decreased proliferative capacity of various differentiating cell populations (Buttitta & Edgar 2007; Jasper et al. 2002; Xia et al. 2006; Bendall et al. 2003). Conversely, exposure to pro-proliferative signals can inhibit differentiation (Rowitch et al. 1999).

To progress through the cell cycle, cells must pass a series of tightly regulated checkpoints, controlled in large part by cyclin dependent kinase (CDK) activity. These serine/threonine protein kinases, which require the presence of cyclin proteins to be active, phosphorylate various proteins at specific points of the cell cycle, causing transcriptional changes that allow the cell to continue through the cell cycle (Vermeulen, Van Bockstaele & Berneman 2003). Alterations in CDK activity can lead to deregulated cell checkpoints, increasing the chances of oncogenic transformation within a cell; therefore CDK activity must be regulated at multiple levels. Specific cyclin-CDK complexes govern each cell cycle phase and
while CDK levels remain relatively stable throughout, cyclins are synthesized and degraded at defined times in the cell cycle. CDK inhibitors (CKI) prevent cell cycle progression by inactivating CDK-cyclin complexes or preventing them from forming (Bertoli, Skotheim & Bruin 2013; Vermeulen, Van Bockstaele & Berneman 2003). Because differentiation of a cell in almost all cases requires an exit from the cell cycle, genes that promote differentiation often act directly or indirectly to inhibit cyclins, or to activate CKIs. In either case, CDK activity is downregulated and the cell cycle is arrested (Buttitta & Edgar 2007).

The Dlx gene family

The Distal-less homeobox (Dlx) gene family encodes a group of homeodomain-containing transcription factors vital to embryonic development. The homeodomain is a 60 amino acid DNA-binding domain encoded by the 180 bp region called the homeobox (Gehring, Affolter & Bürglin 1989). Homeobox-containing genes have been found in every eukaryote studied and Dlx genes are present in all members of the phylum Chordata. The most primitive chordates contain only one Dlx gene, while vertebrates have evolved to have at least six Dlx/DLX genes, organized into three first-order paralogous pairs: Dlx1&2; Dlx3&4 and Dlx5&6, located on three different chromosomes (Figure 1.1) (Holland et al. 1996; Merlo et al. 2000; Sumiyama, Irvine, & Ruddle 2002). Chick orthologues of all but one murine Dlx gene have been identified; the expression of a functional Dlx4 gene in chicks has yet to be confirmed (Table 1.1) (Ferrari et al. 1995; Pera & Kessel 1999; Brown, Wang, & Groves 2005; Stock et al. 1996). In all of these species, both members of each bigene pair show an overlap in gene expression, likely due in part to shared cis-acting regulatory elements found in the intergenic region of each pair (Ghanem et al. 2003; Birnbaum et al. 2012). Single versus double knockout studies have shown that disturbance of the expression of only one of the gene pair members


causes a less severe phenotype than when both pair members are affected, suggesting that some functional redundancy exists within first-order pairs (Robledo et al. 2002; Depew et al. 2005).

**Developmental roles of Dlx genes in the control of proliferation and differentiation**

Vertebrate Dlx family members are orthologues to the single Distal-less (Dll) gene in Drosophila, a gene considered a ‘master regulator’ of ventral appendage development and important in antennal specification and patterning (Dong, Dicks, & Panganiban 2002; Panganiban 2000). Similarly, vertebrate Dlx genes are vital to craniofacial patterning and skeletal development, as well as in neural development and the development of some sensory organs, including the inner ear (Jeong et al. 2008; Robledo et al. 2002; Kraus & Lufkin 1999). Early in development, Dlx genes are expressed in the forebrain, various areas of the surface ectoderm and in the cranial neural crest (CNC) (Zerucha et al. 2000; Puelles et al. 2000; Depew et al. 1999; Pera & Kessel 1999; Acampora et al. 1999). CNC cells migrate to populate the first and second pharyngeal arches (PA1 and PA2), primordial structures which give rise to craniofacial components including the jaw and Reichert’s cartilage (Kulesa and Fraser 2000; Depew, Lufkin, & Rubenstein 2002). In the murine pharyngeal arches, Dlx gene pairs are expressed in an overlapping nested pattern along the dorsoventral axis, with each gene from one pair being expressed with similar spatial and temporal organization (Qiu et al. 1997; Robinson & Mahon 1994; Depew et al. 1999). Later in development, Dlx genes are expressed in differentiating skeletal elements, developing teeth and hematopoietic stem cells (Depew et al. 2002; Acampora et al. 1999; Ryoo et al. 1997; Ferrari et al. 1995; Shimamoto et al. 1997).
Figure 1.1 Chromosomal organizations of murine Dlx gene family members

*Dlx* genes are organized on three different chromosomes, with first order paralogues residing on the same chromosome (e.g., *Dlx5/Dlx6*). This figure represents the murine *Dlx* gene family organization. Corresponding human chromosomes are as follows: chromosome 2 (*Dlx1/Dlx2*); chromosome 7 (*Dlx6/Dlx5*); chromosome 17 (*Dlx4/Dlx3*).
Table 1.1 Species-specific differences in Dlx family nomenclature

Alternative/isoform names are indicated in brackets. A functional Dlx4 has not yet been identified in chicks.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Human</th>
<th>Chick</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dlx1</td>
<td>DLX1</td>
<td>Dlx1</td>
</tr>
<tr>
<td>Dlx2</td>
<td>DLX2 (TES10)</td>
<td>Dlx2</td>
</tr>
<tr>
<td>Dlx3</td>
<td>DLX3</td>
<td>Dlx3</td>
</tr>
<tr>
<td>Dlx4 (Dlx7)</td>
<td>DLX4 (DLX7, DLX8, BP1)</td>
<td>?</td>
</tr>
<tr>
<td>Dlx5</td>
<td>DLX5</td>
<td>Dlx5</td>
</tr>
<tr>
<td>Dlx6</td>
<td>DLX6</td>
<td>Dlx5</td>
</tr>
</tbody>
</table>
In general, *Dlx* genes are known to act as transcriptional activators, activating lineage-specific genes to promote differentiation of progenitor cells. As promoters of differentiation, they tend to have a negative effect on cell division, however whether this is a direct physiological role of these proteins has not been established. *Drosophila Dll*, on the other hand, has been shown to directly interact with *Drosophila* DNA replication-related element-binding factor (dDREF), an important proliferation related protein (Hayashi et al. 2006). dDREF is a transcription factor that binds to a DNA replication-related element (DRE) in genes encoding proteins such as proliferating cell nuclear antigen (PCNA) and DNA polymerase α, both important in DNA replication (Hochheimer et al. 2002; Takahashi et al. 1996). Through a series of glutathione-S-transferase pull down assays, it was found that the N-terminus of Dll interacts with a 110 amino acid sequence near the N terminus of dDREF, inhibiting its DNA binding ability (Hayashi et al. 2006). dDREF can also be repressed by other proteins with pro-differentiation capacities, and is likely a key target in the proliferation/differentiation switch in *Drosophila* (Matsukage et al. 2008). A human homologue of dDREF, hDREF, has been found to promote cellular proliferation in mammals, but the potential ability of any vertebrate Dlx proteins to interact with hDREF remains unexplored (Yamashita et al. 2007).

Dlx proteins are known to interact however, with the pro-proliferative homeodomain-containing Msh homeobox (Msx) proteins. Msx proteins promote the proliferation of precursor cranial neural crest (CNC) cells in the pharyngeal arches, while preventing their terminal differentiation (Mina et al. 1995; Alappat, Zhang & Chen 2003). Conversely, *Dlx* gene family members promote differentiation in the pharyngeal arches, at the expense of proliferation. The combination of Dlx proteins present in the pharyngeal arches, along with the presence or absence of Msx proteins and other Dlx inhibitors seems to determine the proliferative or differentiative
state of the CNC cells as the pharyngeal arches expand along the dorsoventral axis (Medeiros & Crump 2012). Dlx and Msx proteins have been found to interact, and exhibit reciprocal inhibition, providing evidence for a functional antagonism between these two opposing proteins in the control of proliferation and differentiation (Newberry, Latifi & Towler 1998; Zhang et al. 1997; Bendall & Abate-Shen 2000).

Interaction between Dlx and Msx proteins may also play a role during osteoblastic differentiation. Dlx5 is known to promote osteoblastic differentiation downstream of bone morphogenic protein (BMP)-2 induction by directly upregulating bone-specific genes including osterix (Osx) and osteocalcin (Osc) and promoting the transcription of Runx2, another transcription factor vital to the differentiation of osteoblasts (Kim et al. 2004; Ulsamer et al. 2008; Hassan et al. 2004; Lee et al. 2003). Msx gene family members Msx1 and Msx2 have both been shown to prevent differentiation and moderately promote the proliferation of osteoblasts via the upregulation of cyclin D1 (Hu et al. 2001). Dlx5 and Msx2 have been shown to antagonize each other’s functions, possibly by competing for shared binding sites in promoters of target genes, or through protein-protein interactions that affect each others’ abilities to act upon a target (Hassan et al. 2004; Ryoo et al. 1997; Newberry, Latifi & Towler 1998). In this way, Dlx5 expression could interfere with Msx2-mediated upregulation of cyclin D1, stopping cell cycle progression and allowing the cell to differentiate.

Dlx5 seems to play an important role in the proliferation/differentiation transition in chondrocytes, evidenced by its strict spatial and temporal expression pattern. In the growing limb of developing embryos, mesenchymal cells condense and differentiate into chondrocytes which proliferate rapidly in columns to drive elongation of the prospective bone. However, as the
template grows, chondrocytes in the centre of the prospective bone (epiphysis) receive signals to exit the cell cycle and differentiate into hypertrophic chondrocytes, which eventually produce a mineralized matrix. This leads to a distinct zone of proliferative chondrocytes and zones of increasing stages of chondrocyte differentiation, with the proliferative ability of these chondrocytes inversely related to their stage of differentiation (Figure 1.2). Dlx5 is first expressed in the precartliaginous condensations within the developing limb and is later re-expressed in chondrocytes entering the prehypertrophic zone. Its lack of expression in the proliferative zone of chondrocytes suggests that Dlx5 expression is incompatible with proliferation (Bendall et al. 2003; Hsu et al. 2006; Ferrari & Kosher 2002). Furthermore, cartilage-specific overexpression of Dlx5 in the developing limbs of mice causes a reduction in the length of the proliferative zone concomitant with an expanded zone of hypertrophic chondrocytes (Chin et al. 2007). Similarly, retroviral infection of chick limb buds with a Dlx5-containing vector decreases proliferation and expands the zone of hypertrophic chondrocytes into the region normally containing proliferating chondrocytes, suggesting that Dlx5 promotes chondrocyte differentiation at the expense of proliferation (Ferrari & Kosher 2002). Consistent with these observations, Dlx5 overexpression in primary fibroblasts in vitro reduces proliferation and stimulates chondrogenic differentiation (Bendall et al. 2003).
Figure 1.2 Schematic model of Dlx5 expression in differentiating chondrocytes

During endochondral ossification of long bones, detection of Dlx5 coincides with the onset of differentiation and a decreased proliferative capacity. Overexpression of Dlx5 increases the number of hypertrophic chondrocytes, at the expense of the proliferative zone of chondrocytes, and expands the area in which markers of chondrocyte maturation can be detected.
Evidence that Dlx5 plays a direct role in cell cycle control

The concept of a dual role for homeobox genes in the switch from proliferation to differentiation is not without precedent. The *Drosophila* homeodomain protein Prospero (Pros) is required for neuronal differentiation, directly activating genes required for neuronal cell specification. Neural stem cells undergo asymmetric cell division, giving rise to one self-renewing daughter cell, and one ganglion mother cell (GMC) which gives rise to two terminally differentiated neurons. In *Drosophila* lacking *prospero* expression, the GMC fails to differentiate and instead continues to self-renew (Choksi et al. 2006; Li & Vaessin 2000). Pros is able to bind near the *Cyclin E* (*CycE*) gene, the central regulator of cell cycle in *Drosophila*. Overexpression of *Pros* has been shown to reduce transcript levels of *CycE*, while in *Pros* knockout embryos, *CycE* transcript levels are increased. This provides strong evidence that Pros directly represses transcription of *CycE*, thereby giving it a dual role in the switch from proliferation to differentiation (Choksi et al. 2006). Its mammalian homologue, *Prox1*, also regulates both processes (Dyer et al. 2003). These experiments demonstrate that homeobox genes can have direct transcriptional effects on both differentiation-related and proliferation-related genes.

Whether Dlx5 or Dlx6 are similarly able to directly affect proliferation-related genes during development is unknown, however cancer-related studies have given some evidence to suggest that Dlx5 is able to directly affect cell cycle control pathways. The first evidence of *Dlx5* involvement in carcinogenesis was when a cDNA oligonucleotide array analysis uncovered that *Dlx2* and *Dlx5* were overexpressed in a variety of small cell lung cancers (Pedersen et al. 2003). In another experiment, targeted disruption of *DLX5* expression in non-small cell lung cancer cells through siRNA knockdown of *DLX5* led to an inhibition of cell proliferation (Kato et al. 2008). The mechanisms behind this proliferative inhibition, however, were not explored.
Another link to oncogenic control of proliferation by Dlx5 was made in 2008 when it was discovered that a chromosomal rearrangement in mouse lymphomic T cells placed a T-cell receptor β (Tcrβ) enhancer near the Dlx5/6 locus (Tan et al. 2008). These potent enhancer elements are commonly found to be translocated near regulatory genes involved in cell proliferation, survival and differentiation, generating oncogenes in T and B cells (Look 1997). In this case, the Tcrβ enhancer significantly upregulated Dlx5 expression in these cells, leading to increased cellular proliferation (Tan et al. 2008).

Further investigation has revealed that increases in proliferation mediated by Dlx5 may be due to its ability to bind to, and activate the transcription of c-Myc, a common oncogene involved in cell cycle control. In lung cancer cells that normally overexpress DLX5, such as NCI-H322M, HCI-H520 and NCI-H23, siRNA knockdown of DLX5 caused a decrease in c-MYC mRNA levels and a decrease in cell expansion relative to cells containing a control siRNA. Conversely, overexpression of DLX5 in Jurkat cells, which do not normally express DLX5, promoted cell proliferation. Gel-shift assays confirmed that DLX5 can bind to the proximal c-MYC promoter and DLX5 overexpression increased promoter activity as measured by a dual-luciferase transcription assay (Xu & Testa 2009). Interestingly, DLX4 has also been shown to regulate c-MYC activity as well as to block growth inhibition by TGF-β, an important regulator of proliferation, apoptosis and differentiation (Trinh, Barengo & Naora 2011). Overexpression of DLX4 promotes cell proliferation in ovarian cancer cell lines both in vitro and in vivo (Hara et al. 2007). In contrast to DLX5, c-MYC regulation has been implicated as a normal physiological role of DLX4 during development (Shimamoto et al. 1997).

Other studies have indicated that transcriptional control of c-Myc is likely not the only way Dlx5 can affect the cell cycle. DLX5 is overexpressed in a variety of human cancer cell lines...
including breast, lung and ovarian cancers (Tan et al. 2010). In ovarian cancer cell lines in which 
\textit{DLX5} is overexpressed, shRNA knockdown of \textit{DLX5} interfered with cell cycle progression, and led to a decrease in cyclin A, B1, D2 and E1 expression. In these ovarian cancer cells, DLX5 was able to bind to the promoter of \textit{Insulin receptor substrate-2 (IRS-2)} and activate its transcription; knockdown of \textit{DLX5} reduced IRS-2 protein levels (Tan et al. 2010). \textit{IRS-2} can promote cellular proliferation in part through the transcriptional activation of cyclins, therefore the ability of Dlx5 to affect proliferation in the cancer cells in which it is overexpressed could also be indirectly mediated by \textit{IRS-2} activation (Wu et al. 2009; Sun et al. 2011; Fatrai et al. 2006; Wu et al. 2010).

**Pro-proliferative roles of Dlx5 and Dlx6 during development**

Although the pro-proliferative effects of \textit{Dlx5} misexpression in cancer cells seem at odds with the antiproliferative effects observed during cell differentiation in development, evidence exists to suggest that \textit{Dlx5} can have varying effects on proliferation depending on the context of its expression. \textit{Dlx5} is expressed in certain specialized epithelial cell populations in the limb and inner ear during development, but does not promote their differentiation. In fact, expression of \textit{Dlx5} seems to be essential for the continued proliferation of these cells. In chick limb buds, \textit{Dlx5} is expressed in the apical ectodermal ridge (AER), a specialized epithelium at the distal end of the limb bud that signals to the underlying mesenchymal tissue to remain undifferentiated and proliferative (Ferrari et al. 1995; Sun, Mariani & Martin 2002). In mice lacking \textit{Dlx5} and its functionally redundant paralogue \textit{Dlx6}, a decrease in proliferation and absence of normal AER expression markers in the medial portion of the AER led to a failure of medial digits to form (Robledo et al. 2002). Inner ear defects were also seen in \textit{Dlx5/6}−/− mice. Structures of the inner ear originally develop from a thickening of surface ectoderm called the otic placode near the hindbrain. The otic placode invaginates to form an epithelial-lined otic vesicle, the cells of which
undergo a period of proliferation before differentiating into the many diverse cells that make up the inner ear (Torres & Giráldez 1998). During normal development, *Dlx5* and *Dlx6* are expressed first in the otic placode and expression continues in the otic vesicle, eventually becoming restricted to the developing vestibular portion of the inner ear. In mouse embryos lacking *Dlx5* and *Dlx6* expression, reduced proliferation of the otic epithelium caused a smaller otic vesicle and the vestibular portion of the inner ear was completely absent (Robledo & Lufkin 2006).

A context-dependent difference in the control of proliferation has been demonstrated by a variety of other homeobox genes. For example, *HOXA10*, a homeobox gene from the *Hox* gene family, can support cell cycle arrest and differentiation in leukemic human cells in culture, while in primary mouse bone marrow cells in culture it promotes proliferation (Bromleigh & Freedman 2000; Bjornsson et al. 2001). The level of HOXA10 present in a cell could be a determinant of whether its effect on proliferation is positive or negative. In haematopoietic stem cells exposed to increasing levels of HoxA10, moderate levels of HoxA10 caused proliferation to increase while higher levels of HoxA10 caused a decrease in proliferation (Magnusson et al. 2007). Members of the *Caudal-type homeobox* (*Cdx*) gene family, which are important during embryonic development in axial and gut endoderm patterning have also been found to have context-dependent effects on cell proliferation (Beck and Stringer 2010). In adult rat colon cells, Cdx1 stimulates proliferation and migration; in colon cancer cells, CDX1 has been shown to have a positive effect on cell proliferation, mediated by the activation of PCNA (Soubeyran et al. 1999; Oh et al. 2002). However, in other studies involving colon cancer cells, CDX1 inhibits cell proliferation through the inhibition of cyclin D1, D3, A and B and a decrease in the transcriptional activity of *c-MYC* (Lynch et al. 2003; Guo et al. 2004).
Rationale

As evidenced above, the control of proliferation by homeobox genes is complicated and involves many factors; further research is required to truly understand how they function in this role. Full understanding of the molecular interactions involved in cellular processes is an important component of basic scientific and health research and understanding how genes such as *Dlx5* and *Dlx6* normally function during development can give clues as to how their misregulation contributes to disease progression. It is useful to study both of these genes, as they have been shown to exhibit functional redundancy during development. Determining the contexts in which they display functionally equivalent effects is useful in advancing our understanding of how *Dlx* genes act, especially when expressed in overlapping domains, as is the case in the pharyngeal arches. Here, we build upon previous reports of proliferative control by *Dlx* genes in cell types of different origins, with or without the capacity to differentiate. Our goal is to determine how the cell cycle is affected by Dlx5 and Dlx6 in order to determine possible transcriptional targets for their role in controlling proliferation.

Hypothesis

The physiological effects of *Dlx5* and *Dlx6* are, in part, mediated through regulation of cell cycle control genes.
CHAPTER 2: MATERIALS AND METHODS

Embryos

Fertile Plymouth Barred Rock chicken eggs were obtained from Arkell Poultry barn (Guelph, ON) and incubated at 38°C for 4 days. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Limb buds and pharyngeal arches of stage 22-24 embryos were dissected and digested using 0.25% trypsin with EDTA (Hyclone). Digested tissue was trituated with a Pasteur pipette which had been passed through a flame to reduce the inside diameter. Tissue was trituated until a single cell suspension was observed microscopically, and then media was added. Cells were counted and then spun at 1 000 rpm in an IEC Centra CL2 centrifuge with a 235 rotor (Thermo) for 5 minutes before being resuspended at a concentration of 2x10^7 cells/mL.

Cell Lines and Reagents

Subconfluent HeLa, DF-1, HEK-293T, C2C12 and primary limb bud cells were maintained at 37°C and 5% CO₂ in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% (HeLa, DF-1, HEK-293T, primary cells) or 20% (C2C12) fetal bovine serum as well as 100 U/mL penicillin, 100 μg/mL streptinomycin and 2 mM L-glutamine.

Resazurin Blue Cell Viability Assay

Transfected cells were seeded into a 96-well plate at equal density in 100 μL of supplemented DMEM. Each day, 100 μL of 0.05 mM resazurin blue (Sigma) in supplemented DMEM was added to 3 wells. Fluorescence was measured at Ex516λ and Em590λ in a microplate fluorescence reader immediately after resazurin blue addition to obtain a background fluorescence reading. A second measurement was taken after 4 hours of incubation at 37°C to
allow metabolically active cells to convert resazurin to the fluorescent compound resorufin. Measurements were repeated every 24 hours for 5 days. Background fluorescence readings were subtracted from the reading taken 4 hours after incubation and changes in fluorescence over time were normalized against the reading taken at day 0.

**Caspase-3 Activation Assay**

Adherent cells were trypsinized, counted and incubated in cell lysis buffer (50 mM HEPES, pH 7.4; 0.1% CHAPS; 0.1 mM EDTA; 1 mM DTT) at a density of 1.5x10^6 cells/50 μL on ice. Following 30 minutes of incubation, extracts were spun at 20 000 xg for 10 minutes at 4°C. Caspase activity was measured by mixing 25μL of cell extract with 75μL of reaction buffer (50 mM HEPES, pH 7.4; 100 mM NaCl; 0.1% CHAPS; 10 mM DTT; 1 mM EDTA; 10% glycerol) and 100 μL of 60 μM fluorogenic caspase-3 substrate (Ac-DEVD-AMC). The amount of AMC released was measured at 37°C every 5 minutes for 90 minutes using a BIO-TEK Flx800 microplate fluorescence reader (BIO-TEK, Winooski, VT) with 360/40 nm Fluorescence Filter (BIO-TEK) for excitation and 460/40 nm Fluorescence Filter (BIO-TEK) for emission. Protein concentration for each sample was determined using BCA protein assay kit (Pierce) according to the manufacturer’s instructions using a spectrophotometer at a wavelength of 454 nm. Using these protein concentrations, the relative fluorescence units (RFU) released per minute, per μg of protein added was determined.

**Immunoblot analysis**

Cells were collected in chilled DPBS and lysed in high salt lysis buffer (50 mM Tris, pH 8; 500 mM NaCl; 1% Triton X-100) with protease inhibitors (Complete Mini Protease Inhibitor; Roche) with sonication. Protein concentration was quantified using a BCA protein assay kit. Protein was separated by SDS-PAGE in a 13% poly-acrylamide gel then transferred to a PVDF
membrane. Membrane was blocked for at least 1 hour in 5% skim milk powder in PBS containing 0.1% Tween-20 (PBT). Membrane was incubated for at least 2 hours in 9E10 mouse-α-MYC primary antibody at a dilution of 1:500 in blocking solution, followed by 1 hour in 1:10 000 horseradish peroxidase-conjugated goat-α-mouse secondary antibody (Bio-Rad) in blocking solution. Membranes were reprobed with mouse anti-β-actin antibody (Genetex) to confirm equal sample loading. Membranes were incubated for 2 hours with primary anti-β-actin antibody diluted 1:2000 in blocking solution followed by 30 minutes in 1:10 000 horseradish peroxidase-conjugated goat-α-mouse secondary antibody in blocking solution. Immunoreactive bands were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and imaged with a Bio-Rad molecular Imager Chemi-Doc XRS+ and ImageLab software (Bio-Rad).

**Transfection**

DF-1, HeLa and HEK293T cells were seeded 24 hours prior to transfection then transfected at subconfluency with polyethylenemine (PEI) at a ratio of 4.5 μg PEI to 1 μg plasmid DNA. C2C12 and primary chick embryonic cells were transfected in suspension using Effectene Transfection Reagent (Qiagen) with EC buffer supplemented with 0.4 M trehalose, at a ratio of 6 uL Effectene per μg of plasmid DNA. 24 hours post transfection, Geneticin (G418) (BioShop) was used to select for pcDNA3-transfected cells and puromycin (Fisher Scientific) was used to select for LZRS-transfected cells. No selection was used on RCAS(A)-transfected cells.

**Coverslip preparation**

Autoclaved glass coverslips were coated with 0.1 M poly-D-lysine for 30 minutes and washed twice with sterile deionized water and once with DMEM before cells were seeded.
**EdU Incorporation Assay**

from Life Technologies. Cells were transfected in suspension directly onto poly-D-lysine coated coverslips and allowed to settle for 24 hours before EdU incubation. Transfected cells were incubated for various times with 10 μM EdU in DMEM at 37°C, 5% CO₂, fixed in 3.7% formaldehyde in PBS and permeabilized using 0.5% Triton-X-100 in PBS. EdU was detected with a Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Life Technologies) as per the manufacturer’s instructions. Indirect immunodetection was performed after EdU detection.

**TUNEL Assay**

Cells were transfected in suspension directly onto poly-D-lysine coated coverslips and collected 24 hours post transfection. Cells were fixed in 3.7% formaldehyde in PBS and permeabilized using 0.25% Triton-X-100 in PBS. DNase I was used as a positive control for detection of DNA breaks. DNA breaks were detected with a Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay for microscopy (Life Technologies) as per the manufacturer’s instructions.

**Indirect Immunofluorescence**

Cells were fixed and permeabilized as per experimental requirements (EdU incorporation assay and TUNEL assay). After the Click-iT® reaction, cells were washed twice in PBS supplemented with 10 mM glycine (PBS-G). Myc-tagged Dlx proteins were detected with 9E10 mouse antibody diluted 1:50 with 3% bovine serum albumin (BSA) in PBS-G for 2 hours at room temperature in the dark. Coverslips were washed twice with PBS-G then incubated for 45 minutes with secondary FITC-conjugated goat anti-mouse antibody (Genetex) diluted 1:100 with 3% BSA in PBS-G. Coverslips were then washed 3 times with PBS-G and incubated for 30 minutes in 5 µg/mL Hoechst 33342 at room temperature in the dark. Coverslips were washed twice with PBS and once with sterile deionized water and allowed to dry before mounting in 1,4-
diazabicyclo[2.2.2]octane (DABCO) solution (10mg/mL DABCO in 1:9 PBS: Glycerol; 0.02% sodium azide).

**Dual Luciferase Transcription Assays**

Cells were transfected in 12-well dishes with 16 ng pRL-SV40, a *Renilla* luciferase under the control of an SV40 enhancer/promoter (Promega), and 400 ng of pSNM-luc, a *pGL2* firefly luciferase reporter under control of the proximal *c-MYC* promoter (Facchini et al 1997), and varying amounts of *pcDNA3* plasmids encoding Dlx proteins or the vector alone (See Figure 2.1). 24 hours post transfection (or 48 hours for primary cells), cells were scraped into chilled DPBS and centrifuged for 1 minute at 960 xg. Cells were resuspended in passive lysis buffer (PLB) (Promega) and incubated on ice for 30 minutes. Luciferase levels were detected using the Dual-Luciferase® Reporter Assay System (Promega), using a Turner TD 20e luminometer. 25 μL of Luciferase Assay Reagent II was added directly to each sample and firefly luciferase activity was quantified. 25 μL of Stop and Glo® was added directly to the same tube to quench the firefly luciferase reaction and quantify *Renilla* luciferase activity. The total amount of *pcDNA3* plasmid was maintained at an equal level across different levels of effector plasmid to account for any effect the presence of a *pcDNA3* plasmid may have on the cells (Figure 2.2).

**Flow Cytometry**

Transfected cells were trypsinized and counted 24 hours post seeding. Cells were centrifuged at 900 xg and resuspended at a concentration of 1x10⁶ cells/mL in DPBS. Cells were centrifuged again at 900 xg and fixed with chilled (-20°C) 70% ethanol with vortexing. Cells were then incubated at -20°C for at least 24 hours then pelleted at 3000 xg. Cell pellets were washed twice with PBS then resuspended in 50 μL of 100 μg/mL RNase A (per 1x10⁶ cells) and incubated at room temperature for 10 minutes. 400 μL of 50 μg/mL propidium iodide (PI) in
PBS was added (per 1x10^6 cells) and incubated for 30 minutes in a 37°C water bath. DNA content was determined using a Beckman Coulter Cytomics FC 500MPL flow cytometer at excitation of 488. FL1 (Em 525) was used to detect FITC fluorescence and FL3 (Em 610) was used to detect PI. FL3 histograms were analyzed using the MultiCycle AV DNA analysis software (Phoenix Flow Systems) available in the FCS Express 4 Plus-Research Edition program (De Novo Software). The histogram was fit with the SL G21 S0 one cycle fitting model. G2/G1 ratio was set at 1.93 and background was removed before G1, S and G2 DNA content was analyzed.

**Statistical Analysis**

All statistical analysis was performed using Prism (GraphPad). All linear increases in growth curves and cumulative labeling EdU incorporation assays were fit with a linear regression model, considering each replicate Y value as an individual point. The slopes of any 2 lines were compared with a two-tailed test of the null hypothesis that the slopes were identical. A p value less than or equal to 0.05 was interpreted as a rejection of the null hypothesis. If the p value was greater than 0.05, the intercepts of the slopes were compared. A second p value was calculated testing the null hypothesis that the lines were identical. A p value of less than or equal to 0.05 was interpreted as a rejection of the null hypothesis. Two-way analysis of variance (ANOVA) was performed on any data which had multiple variables affecting the outcome, including the time-point caspase-3 activation assay, 4hr EdU incorporation assay and TUNEL. Other caspase-3 activation assays were analyzed using a one-way ANOVA. Flow cytometry data was compared with unpaired t-tests, using the Holm-Sidak method with an alpha of 5% to correct for multiple comparisons. Dose-responsiveness in transcription assays was determined for each Dlx protein separately using a one-way ANOVA on relative activation levels while the
normalized luciferase values were used in a paired \( t \)-test to compare basal activation to maximum activation. In all of these instances, a \( p \) value less than or equal to 0.05 was considered statistically significant.
Figure 2.1: Dual-luciferase reporter system
Schematic representation of luciferase assay
**Figure 2.2: Plasmid mixes for dual-luciferase reporter system**

*pSNM-luc* (firefly luciferase reporter) and *pRL-SV40* (*Renilla* luciferase control) plasmid concentrations remained constant across all experiments. Firefly luciferase levels were normalized against background levels measured in populations containing an equal amount of empty *pcDNA3* plasmid as the total (*pcDNA3* with effector + empty *pcDNA3*) in that population. All plasmid amounts are in nanograms.

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CHAPTER 3: RESULTS

3.1 Effects of Dlx proteins on cell growth

Dlx5 has been shown to play an important role in BMP-2 mediated differentiation of the multipotent myoblastic C2C12 cell line into osteogenic cells (Lee et al. 2003). Additionally, stable transfection of C2C12 cells with Dlx5 alone has been shown to stimulate alkaline phosphatase (AP) activity, a marker of differentiation (Kim et al. 2004). We transfected proliferating C2C12 cells with pcDNA3 plasmids containing myc-tagged Dlx5- or Dlx6-encoding genes, or with a pEGFP-C1 plasmid encoding green fluorescent protein (GFP) and used G418 to select for plasmid containing cells. Selected cell populations were then seeded into a 96-well plate at equal density and relative changes in the number of metabolically active cells over time was measured (Figure 3.1.1.A, 3.1.1.C). Over the course of 4 days, Dlx-transfected cell populations expanded at a slower rate in comparison to the vector control population (Figure 3.1.1.B). Cells transfected with the GFP-containing plasmid showed no significant change in growth rate when compared to the pcDNA3-containing control population (Figure 3.1.1.D), suggesting that the effects of the Dlx proteins are not due to an overall increase in protein expression in the cell, but are specifically an effect of Dlx protein expression. To verify that the reduced population expansion over time was not a result of an increase in cell death, we collected extra cells at the time of seeding the proliferation assay to measure activated caspase-3 levels. Caspase-3 is an effector caspase of the apoptosis-induced cysteine protease family. Normally inactive in healthy cells, the presence of activated caspase-3 indicates that a cell is irreversibly committed to apoptosis (Riedl & Shi 2004). Therefore, an increase in active caspase-3 in a cell population is representative of an increase in the apoptotic index of that population. At the time of seeding the proliferation assay (day 0), no significant increase in caspase-3 activation
was seen in Dlx-transfected populations (Figure 3.1.2.A). While these data did not reveal increased apoptosis at the time of seeding, an increase in apoptotic activity may have been triggered at later time points. Additionally, we may have underestimated apoptotic levels by collecting only the adherent cells, because caspase-3 activation can cause accelerated detachment of adherent cells (Turner et al. 2003). Therefore, we wanted to test caspase-3 activation levels in both floating and attached cells. To achieve this, we seeded transfected and selected C2C12 cells into 6-well plates and allowed them to settle for 24, 48 or 72 hours. At each of these times, both floating and adherent cells were collected and activated caspase-3 levels were assessed. Once again, no significant change in activated caspase-3 levels was detected (Figure 3.1.2.B).

At the time of seeding, we also collected extra cells to perform immunoblot analysis in order to verify expression of Dlx proteins. We were consistently unable to confirm protein expression, despite seeing an obvious effect on cell growth in Dlx-transfected populations. Because C2C12 cells were being transiently transfected, we hypothesized that Dlx expression was being lost or downregulated over the extended selection period. To confirm, we transfected C2C12 cells and collected 24 hours post transfection with no selection, and 5 days post transfection, with 4 days of selection. As expected, Dlx5 and Dlx6 expression was detectable at the 24 hour time point, but undetectable after selection (Figure 3.1.3).
Figure 3.1.1: Dlx proteins reduce the rate of cell accumulation in C2C12 cells.

Proliferating C2C12 cells were transfected in suspension with 4.5 μg of DNA in 6-cm dishes. Cells were maintained at subconfluency and selected over 4 days with 1 mg/mL G418. A mock transfection was used as a selection control. Cells were then seeded at equal density (1x10^4 cells/well) in triplicate per time point in a 96-well plate without selection. (A, C) Relative changes in cell number were measured daily for 5 days using resazurin blue. (B, D) Linear regression analysis was performed on the initial increase in cell numbers, and the slopes of the regression lines were compared using the “Comparison of Regression Lines” function in Prism. The slopes of the Dlx5 and Dlx6 regression lines were determined to be significantly different from the vector control (*p<0.05; Dlx5 p=0.0057; Dlx6 p=0.0245), while the slope of GFP regression line was not (p=0.35).
Figure 3.1.2: *Dlx*-transfected C2C12 populations do not exhibit increased apoptosis

(A) At the time of seeding the resazurin blue viability assay, extra C2C12 cells were collected to assess caspase-3 activation levels (n=5). (B) C2C12 cells were transfected and selected in the same manner as for the proliferation assay (n=2). After 4 days of selection, cells were seeded into 6-well plates, in triplicate for each time point, at a density of 1.9x10^5 cells/well. At 24, 48 and 72 hours-post seeding, floating and adherent cells were collected and centrifuged at 3000 rpm. Bars represent the average caspase activity, +/- SEM (RFU/minute/µg). There was no significant difference in caspase-3 activation in *Dlx5* - or *Dlx6*-transfected populations compared to the vector control at any time point (ANOVA).
To address the issue of losing episomal Dlx expression after extended selection, we used Dlx-containing replication competent avian retroviruses to transfect DF-1 cells. DF-1 cells are embryonic chick fibroblast cells capable of recombinant virus propagation. After replication-competent retroviral RCASBP(A) plasmids are transfected into DF-1 cells, the provirus is reverse transcribed and integrated into the host cell genome. The retroviral genome is then stably transcribed and translated by the host cell machinery and viral proteins are translated and assembled in the plasma membrane. The newly synthesized virions bud from the cell and mature into viral particles, which then infect the surrounding cells. Because the viral particles infect a large proportion of the cell population, selection to enrich the Dlx-expressing population is unnecessary. In this way, although cells with lower levels of Dlx proteins still have a growth advantage, there is still a large population of cells with high levels of expression at the time of seeding. High levels of expression at the time of seeding were confirmed by immunoblot analysis (Figure 3.1.4).

Reduced cell numbers were observed at each time point in Dlx5 and Dlx6 expressing DF-1 populations, compared to the vector control population (Figure 3.1.5.A). The rate of cell accumulation was significantly reduced in each of these populations (Figure 3.1.5.B). To determine whether DNA binding by Dlx proteins is required for growth suppression, we used a mutated Dlx5 gene containing three alanine amino acid substitutions in the homeodomain encoding region (Dlx5HDM). The alanine substitutions render the mutated Dlx5 unable to bind to DNA (Bendall et al. 2003). Unlike Dlx5 and Dlx6, Dlx5HDM did not reduce the rate of expansion in DF-1 cells (Figure 3.1.5.B). Extra cells at the time of seeding were collected to determine activated caspase-3 levels. Again, no significant increase in activated caspase-3 was detected (Figure 3.1.5.C).
Figure 3.1.3: *Dlx* expression is lost after selection in transfected C2C12 cells.

Two 6-cm plates were transfected with pcDNA3 plasmids containing Dlx5, Dlx6 or the vector alone. 24-hours later (1 day post transfection (dpt)), cells from both plates were combined and half were collected and frozen for future immunoblot analysis. The other half was expanded into a 10-cm plate under selection with 1mg/mL G418. Cells were maintained at subconfluency with G418 selection for 4 days and then collected for immunoblot analysis (5 dpt). Samples from both time points were lysed in parallel. M=molecular mass standards.
Figure 3.1.4: Dlx proteins are detectable on day 0 of DF-1 proliferation assay

At the time of seeding the DF-1 resazurin blue cell viability assay, extra cells were collected for immunoblot analysis. Dlx-encoding RCASBP(A) plasmids, or the vector alone, were used to transfect DF-1 cells and no selection was used. M=molecular mass standards.
A:

Fold change in cell number vs. Days Post Seeding for different treatments (Vector, Dlx5, Dlx6, Dlx5HD^M).

B:

Fold change in cell number vs. Days Post Seeding for different treatments (Vector, Dlx5, Dlx6, Dlx5HD^M), with statistical significance indicated by asterisks.

C:

RFU/μg/min for different treatments (Vector, Dlx5, Dlx5HD^M).
Figure 3.1.5: Cell accumulation is significantly reduced in Dlx-expressing DF-1 cells

1x10⁶ proliferating DF-1 cells were seeded into 6 cm dishes and transfected 24 hours later with 4 μg DNA. 24 hours post transfection, cells were expanded to a 10 cm dish. 48 hours later, transfected populations were expanded to 15 cm dishes along with ¼ of a subconfluent untransfected plate and the virus-containing media from the transfected 10 cm dish. 10 mL of fresh media was added. 48 hours later, cells were seeded at equal density (1.2x10⁴ cells/well) in triplicate in a 96-well plate without selection. (A) Relative changes in cell number were measured with a resazurin blue viability assay. (B) Linear regression analysis was performed on the linear increase in cell numbers, and the slopes of the regression lines were compared using the “Comparison of Regression Lines” function in Prism. The slopes of the Dlx5 and Dlx6 regression lines were determined to be significantly different from the vector control (**p<0.0001), while the slope of Dlx5HD⁸ regression line was not (p=0.85). (C) At the time of seeding the resazurin blue viability assay, extra DF-1 cells were collected to assess caspase-3 activation levels (n=3). Bars represent the average caspase activity, +/- SEM (RFU/minute/μg). No significant difference was found in the caspase-3 activity between any cell populations (ANOVA).
Dlx5 and Dlx6 have both been shown to promote chondrogenesis in primary limb bud cultures (Hsu et al. 2006). Additionally, Dlx5 has been shown to inhibit proliferation of primary fibroblasts in vitro and of chondrocytes in the limb in vivo (Newberry, Latifi, & Towler 1998; Zhang et al. 1997; Bendall & Abate-Shen 2000). We dissected stage 22-24 chick limb buds in order to determine the effect of Dlx5 and Dlx6 on their cell growth in vitro. Dissociated limb buds were transfected directly into a 96-well plate and relative changes in cell number were assessed with a resazurin blue cell viability assay. Efficiency of primary limb bud transfections ranged from approximately 15-30%, as determined by detection of β-galactosidase in cells transfected with a CMV-βgal plasmid (data not shown).

A significant effect on cell accumulation was seen in the first 24 hours as Dlx5 or Dlx6 expression resulted in a 24 and 21% reduction, respectively, in the fold change in cell number from time of seeding compared to the vector control population (Figure 3.1.6.A). The rate of change during the initial population expansion was significantly different in the Dlx5 and Dlx6 expressing populations (Figure 3.1.6.B). To assess the effect of Dlx proteins on cell death, dissected stage 22-24 chick limb buds were transfected directly into 6-cm dishes and collected 48 hours post transfection. No change in caspase-3 activity was observed (Figure 3.1.6.C). Similarly, dissected stage 22-24 chick limb buds were transfected directly into 6-cm dishes and collected 48 hours post transfection in order to detect Dlx protein expression. Variable expression levels of Dlx5, and Dlx6 was observed (Figure 3.1.7).

We next wanted to determine the effects of Dlx proteins on cell proliferation in a non-fibroblast cell line. A study that suggests a pro-proliferative role for Dlx5 in cancerous cells provided evidence that Dlx5 promotes proliferation via the upregulation of c-MYC and DLX5 was shown to promote the transcriptional activation of c-MYC in HEK293T cells in this study.
(Xu & Testa 2009). However, the ability of Dlx5 to affect cell growth in HEK293T cells was not investigated. We hypothesized that an overexpression of Dlx5 or Dlx6 in HEK293T cells would promote proliferation. In order to investigate this, transfected HEK293T cells transfected with Dlx5- or Dlx6-containing LZRS plasmids and selected with puromycin, as HEK293T cells are resistant to G418 selection. Cells were seeded into 96-well plates after 4 days of selection and over the course of 4 days, no significant effect on viable cell number was seen in Dlx5 or Dlx6-transfected populations, compared to the vector control population (Figure 3.1.8.A), despite Dlx protein expression at the time of seeding (Figure 3.1.8.B).
Figure 3.1.6: Cell accumulation is reduced in Dlx-expressing primary chick limb bud cells

After primary limb buds were dissociated into single cell suspension and resuspended at a density of 2x10^7 cells/mL, 1.6x10^6 cells were transfected with 3 μg of DNA with Effectene. DNA/cell mixture was then seeded directly into 96-well plates, in triplicate, with 4x10^4 cells/well. No selection was used. (A) Relative changes in cell number were measured with a resazurin blue viability assay. (B) Linear regression analysis was performed on the linear increase in cell numbers, and the slopes of the regression lines were compared using the “Comparison of Regression Lines” function in Prism. The slopes of the Dlx5 and Dlx6 regression lines were determined to be significantly different from the vector control (Dlx5 p=0.024; Dlx6 p=0.025). (C) After primary limb buds were dissociated into single cell suspension and resuspended at a density of 2x10^7 cells/mL, 4.8x10^6 limb bud cells were transfected with 9 μg of DNA with Effectene. DNA/cell mixture was then seeded directly into 6 cm plates. 48 hours later, adherent cells were collected in order to detect active caspase-3 levels (n=3). Bars represent the average caspase activity, +/- SEM (RFU/minute/μg). No significant difference was found in the caspase-3 activity between any cell populations (ANOVA).
Figure 3.1.7: Exogenous Dlx protein expression in transfected primary limb bud cells

After primary limb buds were dissociated into single cell suspension, and resuspended at a density of 2x10^7 cells/mL, 4.8x10^6 of limb bud cells at this density were transfected with 9 μg of DNA with Effectene. DNA/cell mixture was then seeded directly into 6 cm plates. 48 hours later, adherent cells were collected in order to detect exogenous Dlx protein expression. (A) and (B) represent two different limb bud isolations, collected on different days and run separately. M=molecular mass standards.
Figure 3.1.8: Dlx expression does not affect cell accumulation in HEK293T cells

1.2x10⁶ HEK293T cells were seeded into 6cm plates and allowed to settle for 24 hours. Cells were transfected with 10 μg of Dlx-encoding LZRS plasmids, or the vector alone. 24 hours later, cells were expanded to 10 cm plates, under selection with 4 mg/mL puromycin. After 48 hours of selection, cells were seeded at equal density (1x10⁴ cells/well) in triplicate in a 96-well plate without selection. (A) Relative changes in cell number were measured with a resazurin blue viability assay. (B) At the time of seeding resazurin blue cell viability assay, extra cells were collected for immunoblot analysis to verify protein expression. HEK293T cells were transfected with Dlx-containing LZRS plasmids, or the empty LZRS plasmid, and selected with puromycin, as this cell line is resistant to G418. M=molecular mass standards.

(A):

[B]:
3.2 Effect of Dlx proteins on the cell cycle of C2C12 cells

After establishing that Dlx proteins negatively affect the accumulation of cells in a population over time, we wanted to determine how it is acting to slow population growth. Because we are interested in the physiological role of Dlx5 and Dlx6 during development, we chose to focus on the myoblastic C2C12 cell line, which undergoes osteoblastic differentiation in response to Dlx5 overexpression. Because expression of Dlx-containing pcDNA3 plasmids is high one day post transfection (Figure 3.1.3), we decided to look at the effect of Dlx expression on proliferation at this time point. In order to do this, we incubated transfected cell populations with EdU, a thymine analogue, conjugated to an alkyne. The incorporation of this thymine analogue into DNA can be detected by an Alexa Fluor® conjugated to an azide which reacts with the alkyne-conjugated EdU in a copper-catalyzed click reaction (Buck et al. 2008). Because EdU is incorporated into newly synthesized DNA, detection of EdU within a cell indicates that the cell was in the S phase of the cell cycle at some point when EdU was present in the culture.

Transfected C2C12 cells were incubated with EdU for 4 hours and Dlx-positive cells were detected using the same primary α-Myc antibody used for immunoblot analysis and a FITC-conjugated secondary antibody. DNA counterstaining was performed with a Hoechst 33342 dye. At least 200 Dlx-positive cells were counted in each slide and at least 800 Dlx-negative cells were counted on the same slides per experimental replicate. Immunofluorescent cells were counted as positive only where the fluorescence was above background levels as judged from vector-transfected cells (Figure 3.2.1.A). We compared the proportion of EdU-positive cells in the Dlx5 and Dlx6-positive populations to the Dlx-negative cells on the same slide as well as to the pcDNA3-transfected population. Dlx-expressing cells showed a significant decrease (p<0.01, unpaired t-test) in EdU incorporation in comparison to the Dlx-negative cells.
on the same slide, decreasing from approximately 60% incorporation to approximately 20% incorporation. There was no difference in the proportion of EdU-positive cells in the pcDNA3-transfected population when compared to the Dlx-negative cells in the Dlx-transfected population (Figure 3.2.1B).

To ensure that the decrease in EdU incorporation was not due to an increase in cell death in the Dlx-expressing populations, we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect DNA fragmentation, a hallmark of apoptosis. 24 hours post transfection, cells were fixed with formaldehyde and terminal deoxynucleotidyl transferase (TdT) was used to incorporate EdU at the 3’ ends of fragmented DNA. The alkyne-conjugated EdU was detected using an azide-conjugated Alexa Fluor® in a copper-catalyzed click reaction and myc-tagged Dlx proteins were detected via immnofluorescence. Cells were counted as positive for apoptosis where the EdU labelling was above background levels and overlapped, at least in part, with the Hoechst 33342-labelled nuclei (Figure 3.2.3.A). Dlx5 and Dlx6-positive cells did not show an increase in apoptosis compared to the Dlx-negative cells on the same slide, as determined by a 2-way ANOVA (Figure 3.2.3.B).
**B:**

![Image of cell staining with Hoechst 33342, FITC (Dlx), EdU, and Combined images for Dlx5, Dlx6, and Vector conditions.](image)

Bar graph showing the percentage of EdU-positive cells for Vector, Dlx5, and Dlx6 conditions. The graph indicates a statistically significant difference (*) between Dlx- and Dlx+ conditions. The data points are labeled with error bars indicating variability.
Figure 3.2.1: Dlx proteins reduce the labeling index in C2C12 cells 24 hours post transfection

Proliferating C2C12 cells were transfected directly onto poly-D-Lysine-coated coverslips and DNA synthesis was detected 24-hours post transfection using EdU. Cells were incubated with EdU for 4 hours. (A) Representative pictures taken with a 40x objective. Yellow arrows point to Dlx-positive cells that have incorporated EdU during the incubation time. (B) The labeling index (LI) is defined as the ratio of EdU-positive nuclei to total nuclei. Bars represent the average LI, +/- SEM from 3 experiments, with at least 200 Dlx-positive cells counted per experiment. **p<0.01 (Unpaired t-test).
A:

B:
Figure 3.2.3: *Dlx* expression does not increase apoptosis in C2C12 cells 24 hours post transfection

Proliferating C2C12 cells were transfected directly onto poly-D-Lysine-coated coverslips and apoptosis was detected 24 hours post transfection using a TUNEL kit. DNase I treatment was used as a positive control on an untransfected slide to determine level of fluorescence above background at which to count cells as positive for extensive DNA damage. (A) Representative pictures taken with at 40x objective lens. Insets are examples of Dlx-positive, TUNEL-positive cells. (B) The proportion of Dlx-expressing cells undergoing apoptosis was compared to the surrounding Dlx-negative population. Bars represent two experiments +/- SEM, with at least 100 cells counted in each. No significant differences in %TUNEL labeling were detected, as determined by ANOVA.
The conclusions that can be drawn from the results of an EdU-incorporation assay at any one time point are limited as it is a snapshot that is difficult to contextualize without further analysis. A reduction in EdU incorporation in Dlx-expressing cells could be due to a reduction in the proportion of actively dividing cells or a lengthening of the cell cycle; either possibility alone, or a combination of both, could give the same result when looking at only one time point. In order to distinguish between these possibilities, we used a cumulative labeling method to estimate cell cycle length (\(T_c\)) and the growth fraction (GF), defined as the number of cells in a population that are actively undergoing cell division (Nowakowski, Lewin, & Miller 1989). EdU was added to the media of transfected cells and incubated for increasing amounts of time, ranging from 30 minutes to 36 hours. Cells were collected at each time point, and the EdU labeling index of Dlx-positive and Dlx-negative cells was determined by dividing the total number of cells counted by the amount that had incorporated EdU at that time point. The labelling index of both Dlx-positive and Dlx-negative cell populations increased linearly until the GF was labelled. Detailed explanation on the method used to determine GF is provided in the Appendix, Figures A.1 and A.2. While \(Dlx5\) and \(Dlx6\)-expressing cells reached a growth fraction of 76% and 75% respectively, the Dlx-negative populations surrounding each of them exhibited a GF of 94% (Figure 3.2.4). The slopes of each linear increase were determined by applying a linear regression line to all points with a Y value with less than that of the GF. While the slopes of the linear increase were not determined to be significantly different in the Dlx+ populations compared to the Dlx- populations, the length of time required to reach maximum labeling was increased by approximately 6 hours in both the Dlx5- and Dlx6-expressing populations (Table 3.2.1).
Figure 3.2.4 Expression of *Dlx5* or *Dlx6* in a C2C12 cell population decreases the proportion of actively cycling cells

Slopes of the initial linear increases, as determined by linear regression analysis, were compared in Prism, using the “Comparison of Regression Lines” function. Neither Dlx5 (A) nor Dlx6 (B) significantly altered the rate of EdU incorporation, compared to the surrounding Dlx- cells (Dlx5 p=0.07; Dlx6 p=0.14). Growth fraction (GF) was set as the mean of all points following the initial linear increase (see Figure A.4 for details). The elevations of the GF reached by Dlx+ and Dlx- cells were compared using the “Comparison of Regression Lines” function in Prism, and both Dlx5 (A) and Dlx6 (B) were found significantly alter the GF, compared to the surrounding Dlx- cells (Dlx5, Dlx6 p<0.0001).
### Table 3.2.1 Estimation of cell cycle length

Cell cycle length was determined by dividing the maximum labeling index (GF) by the slope of the linear increase, as described previously by Nowakowski et al. 1989.

<table>
<thead>
<tr>
<th>Population</th>
<th>Maximum LI</th>
<th>Slope of Linear Increase</th>
<th>Approximate Tc in hours (GF/Slope)</th>
<th>Time to reach maximum LI (in hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dlx5+</td>
<td>76%</td>
<td>4.20</td>
<td>18h</td>
<td>16h</td>
</tr>
<tr>
<td>Dlx5-</td>
<td>94%</td>
<td>5.60</td>
<td>17h</td>
<td>10h</td>
</tr>
<tr>
<td>Dlx6+</td>
<td>75%</td>
<td>3.73</td>
<td>20h</td>
<td>17h</td>
</tr>
<tr>
<td>Dlx6-</td>
<td>94%</td>
<td>4.99</td>
<td>19h</td>
<td>11h</td>
</tr>
</tbody>
</table>
3.3 Flow Cytometry

We next wanted to determine where in the cell cycle Dlx proteins were having an effect. In order to do this, we used flow cytometry analysis to determine the DNA content within Dlx-transfected and vector-transfected cell populations, and therefore the proportion of cells in each phase of the cell cycle. Proliferating C2C12 cells were transfected and selected in the same manner as for the resazurin blue proliferation assay. After 4 days selection, each cell population was seeded at equal density into 3 separate 6-cm plates. 24 hours post transfection, one plate was collected for analysis and the other two plates were re-seeded at equal density. 48 hours post transfection, one more plate was collected for analysis and the last plate was reseeded for collection 72 hours post transfection. This reseeding was necessary because cell density affects cellular expansion, and therefore the cell cycle profile of a population. No difference in the DNA content was detected in the Dlx-transfected versus the vector-transfected populations at the 24 or 72 hour time points (Figure 3.3.1.A, 3.3.1.C). At the 48 hour time point, a statistically significant reduction in the proportion of cells in S phase was seen, concomitant with an increase in the proportion of cells in the G1/G0 phase (Figure 3.3.1.B). Representative flow cytometry plots from the 48 hour time point are shown in Figure 3.3.2.

Because the extended selection period caused a loss of Dlx expression (Figure 3.1.3), we wanted to analyze DNA content in transiently transfected cells, 24 hours post transfection, using antibodies to detect Dlx proteins. Double labeling of Dlx proteins and propidium iodide was unsuccessful (data not shown). Flow cytometry analysis was then performed on unselected, transiently transfected C2C12 populations 24 hours post transfection. Transfection efficiency was estimated using a GFP-transfected population. At 15% transfection efficiency, no effect was
seen, but at 30% transfection efficiency, there was a 6% increase in the proportion of G1/G0 cells in Dlx-transfected populations in comparison to the vector-control population (Table 3.3.1).
Figure 3.3.1: Dlx5 and Dlx6 have minimal effect on cell cycle after selection period

Transfected C2C12 cells were selected over a period of 4 days and then $8 \times 10^5$ cells were seeded into 6 cm plates. Cells were collected for flow cytometric analysis and extra cells were reseeded at the same density every 24 hours. Bars represent the mean of all experiments +/- SEM. (24 hour n=4; 48 hour n=4; 72 hour n=3) **p<0.0001, unpaired t-test.
Figure 3.3.2 Representative flow cytometry histograms at 48 hours post selection

Propidium iodide fluorescence is shown as histograms, collected by the FC 500 MPL flow cytometry system (Beckman Coulter) using FL3 filter and analyzed using the Multicycle DNA analysis program on FCS Express 4 Plus (Research Edition). Scaling on both axes is linear. A (pcDNA3), B (Dlx5) and C (Dlx6) represent transfected populations from the same experiment, collected and run in parallel.
<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>Dlx5</th>
<th>Dlx6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15% Efficiency:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Live cells in $G_1$</td>
<td>56</td>
<td>58</td>
<td>56</td>
</tr>
<tr>
<td>% Live cells in $G_{2/S}$</td>
<td>44</td>
<td>42</td>
<td>44</td>
</tr>
<tr>
<td><strong>30% Efficiency:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Live cells in $G_1$</td>
<td>47</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>% Live cells in $G_{2/S}$</td>
<td>53</td>
<td>47</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 3.3.1: Proportion of unselected, transfected population in $G_1$ phase of cell cycle

Transfected cells were collected 24 hours post transfection. A C2C12 cell population transfected at the same time with $pEGFP-C1$ was analyzed to estimate the transfection efficiency. Numbers represent one experiment.
3.4 Effect of Dlx proteins on transcription of c-MYC

The first evidence that Dlx5 is able to directly regulate a cell cycle control gene showed that Dlx5 was able to bind to and transcriptionally activate the proximal \( c-MYC \) promoter. Evidence was provided that the regulation of \( c-MYC \) by Dlx5 was the key to its ability to drive proliferation in tumourigenic Jurkat cells (Xu & Testa 2009). We wanted to test whether Dlx5 and Dlx6 were similarly able to affect the transcription of this cell cycle regulator in the cells in which it inhibits cell proliferation. In order to do this we used a firefly luciferase reporter cloned downstream of a proximal \( c-MYC \) promoter containing two high-affinity and two low-affinity Dlx5 binding sites (Figure A.3).

We first validated our system in HeLa cells, by attempting to verify a dose-responsive activation of \( c-MYC \) by Dlx5 and Dlx6. HeLa cells were transiently transfected with increasing amounts of pcDNA3 plasmids containing Dlx5 or Dlx6-encoding genes or the vector alone along with the pSNM-luc proximal \( c-MYC \) reporter luciferase and a Renilla-luciferase plasmid under the control of an SV40 enhancer/promoter as a control. Firefly luciferase values were normalized against the Renilla-luciferase readings and then normalized against the background luciferase activation in cells transfected with the vector alone (Figure 2.1). The proximal \( c-MYC \) promoter appeared to exhibit a dose-responsive activation under the control of Dlx5 or Dlx6, reaching approximately 2-fold activation at maximum reporter activity (Figure 3.4.1.). While this closely matches previously published results in HeLa cells with the same luciferase reporter (Xu & Testa 2009), a paired \( t \)-test determined that the maximum reporter activities in response to Dlx5 or Dlx6 were not statistically different from the basal activation in response to the empty vector and while analysis of variance determined the reporter activity to be significantly dose-dependent in response to increasing levels of Dlx5, it was not significant in response to Dlx6.
Figure 3.4.1: Dlx5 and Dlx6 activate proximal c-MYC promoter in HeLa cells.

HeLa cells were seeded in triplicate into 12-well dishes at 1.2x10^5 cells/well and transfected 24-hours later. Cells were collected 24 hours post transfection and resuspended in 40 μL of PLB. 10 μL of lysed sample was measured per reading. Bars represent fold activation +/- SEM, relative to the vector control, set at 1. Dlx5 n=5; Dlx6 n=3 (all in triplicate). Dose-responsiveness of reporter to Dlx proteins up to maximum activation (indicated by brackets) was tested using one-way ANOVA. Response to Dlx5 was found to be significant (**p=0.0001), and response to Dlx6 was not significant (p=0.092). A paired t-test determined that the maximum reporter activities in response to Dlx5 or Dlx6 were not significant compared to the corresponding levels of empty pcDNA3 in place of effector (Dlx5 p=0.053; Dlx6 p=0.0504).
Figure 3.4.2: Dlx5 and Dlx6 activate proximal c-MYC promoter in C2C12 cells

C2C12 cells were transfected in suspension in 12-well plates and collected 24-hours post transfection. Samples were resuspended in 40 μL of PLB and 10 μL of lysed sample was used per measurement. Bars represent fold activation +/- SEM, relative to the vector control, set at a 1. Dlx5 n=8 Dlx6 n=5. Dose-responsiveness of reporter to Dlx proteins up to maximum activation (indicated by brackets) was tested using one-way ANOVA. Response to Dlx6 was found to be significant (*p=0.0245), while response to Dlx5 was not significant (p=0.11). A paired t-test determined that the maximum reporter activity in response to Dlx6 was significantly different from the corresponding levels of empty pcDNA3 in place of effector (p=0.0014), but the maximum reporter activity in response to Dlx5 was not (p=0.57).
Figure 3.4.3 Dlx5 and Dlx6 activate proximal c-MYC promoter in primary limb bud cells

Dissociated limb bud cells were resuspended in supplemented DMEM at a concentration of 2×10^7 cells/mL and transfected in suspension in 12-well plates. Cells were collected 48-hours post transfection and were resuspended in 10 μL of PLB. 5 μL of lysed sample was used per measurement. Bars represent fold activation +/- SEM, relative to the vector control, set at a 1. Dlx5 n=10 Dlx6 n=6. Dose-responsiveness of reporter to Dlx proteins up to maximum activation (indicated by brackets) was tested using one-way ANOVA. Response to Dlx6 was found to be significant (**p<0.0001), and response to Dlx5 was not significant (p=0.279). A paired t-test determined that the maximum reporter activity in response to Dlx6 was significantly different from the corresponding levels of empty pcDNA3 in place of effector (p=0.0068), but the maximum reporter activity in response to Dlx5 was not (p=0.067).
We then measured c-MYC activation in C2C12 and primary limb bud cells. In C2C12 cells, reporter activity increased in a dose-responsive manner, reaching a maximum activation of 2.9-fold at 320 ng of effector plasmid. This maximum activation was significantly significant, as determined by a paired t-test. Activation by Dlx5 was not statistically significant (Figure 3.4.2). In the primary limb bud cells, reporter activity increased significantly in response to increasing levels of Dlx6 plasmid, reaching 2.3-fold activation at 20 ng. In agreement with the transcription assays performed in C2C12 cells, activation of the proximal c-MYC promoter was not statistically significant (Figure 3.4.3).

We then wanted to see whether Dlx proteins could similarly activate c-MYC expression in HEK293T cells, which did not undergo a Dlx-mediated reduction of cell proliferation. Interestingly, a dose-responsive transcriptional repression of the proximal c-MYC reporter was seen in HEK293T cells transfected with Dlx5 or Dlx6 (Figure 3.4.4). In general, Dlx proteins act as transcriptional activators; however there have been reports of transcriptional repression by DLX1 and DLX2 on the neuropilin-2 promoter (Le et al. 2007). In that instance, the substitution of the N-terminal domain of DLX1 with the transcription activation domain of HSV-1 Viral Protein 16 (VP16) overcame the transcriptional repression and, in fact, activated the transcription of the neuropilin-2 promoter above background levels. A similar VP16 substitution for the N-terminal domain of DLX2 overcame only some of the transcriptional repression (Le et al. 2007). In HeLa cells, we found that VP16 substitution of the N-terminal domain of Dlx5 activated the c-MYC promoter to a higher degree than the regular Dlx5 protein (Figure A.4). The same plasmid was used in HEK293T cells and did not change the dose-responsive repression of the c-MYC promoter. A VP16 substitution for the N-terminal domain of Dlx6 (VDD6) also had no effect (Figure 3.4.5.A). If the C-terminal domain of Dlx5 or Dlx6 was having a dominant
transcriptional effect, then deletion of the C-terminal may abrogate the ability of these proteins to repress the \( c\text{-}MYC \) proximal promoter. We used a Dlx5 homeodomain with a VP16 activation domain substituted for the N-terminal domain of Dlx5, with the C-terminal domain deleted (VDO5). Transcriptional repression of the \( c\text{-}MYC \) reporter was still seen (Figure 3.4.5.B).

Lastly, we used a mutated version of Dlx5 with three alanine substitutions in the N-terminal arm of the homeodomain. This is a similar mutation to the Dlx5HD\(^M\)-containing RCAS(A) plasmid that was shown to abrogate Dlx5 binding to DNA (Bendall et al. 2003). However, the ability of this mutant protein to bind to DNA has not been established. Once again, no change in the transcriptional repression of Dlx5 was seen (Figure 3.4.5.B).
Figure 3.4.4: Dlx5 and Dlx6 repress proximal c-MYC promoter in HEK293T cells

HEK293T cells were seeded in triplicate into 12-well dishes at 1.2x10⁵ cells/well and transfected 24-hours later. Cells were collected 24 hours post transfection and resuspended in 60 μL of PLB. 10 μL of lysed sample was measured per reading. Bars represent fold activation +/- SEM, relative to the vector control, set at 1. Dlx5 n=4; Dlx6 n=3. Dose-responsiveness of reporter to Dlx proteins up to maximum activation (indicated by brackets) was tested using one-way ANOVA. Both Dlx5 and Dlx6 produced a statistically significant dose-responsive transcriptional repression of c-MYC (***p<0.0001, **p=0.008).
Figure 3.4.5: Alterations to Dlx5 and Dlx6 proteins do not reverse dose-dependent transcriptional repression of proximal c-MYC promoter

HEK293T cells were transfected and collected as above. Bars represent fold activation +/-SEM, relative to the vector control, set at 1. (A) VDD5 n=4; VDD6 n=4 (B) VDO5 n=5; Dlx5HDM n=3 (all in triplicate). Dose-responsiveness of reporter to Dlx proteins up to maximum activation (indicated by brackets) was tested using one-way ANOVA. All Dlx proteins produced a statistically significant dose-responsive transcriptional repression of c-MYC (*p<0.05, **p<0.01).
CHAPTER 4: DISCUSSION

Vertebrate Dlx genes and the invertebrate orthologues of Dlx genes such as Distal-less (Dll) in Drosophila encode transcription factors that are vital to appendage patterning and development as well as cell differentiation in a variety of progenitor cell types. There is considerable evidence that Dlx proteins act as transcriptional activators to promote differentiation via the upregulation of lineage-specific genes. As cells differentiate, they lose their ability to divide; therefore, it is not surprising that overexpression of Dlx genes reduces cell proliferation in vivo and in vitro in cells that are able to undergo Dlx-mediated differentiation (Dai et al. 2013; Bendall et al. 2003). However, it is unknown whether Dlx transcription factors have a direct effect on the cell cycle machinery, thereby playing a dual role in the transition of a progenitor cell from proliferation to differentiation or whether the decreased proliferative capacity of a Dlx-expressing cell is an indirect, downstream effect set in motion by initiation of differentiation.

In contrast to its role during development, in a variety of cancer cells, the Dlx transcription factor Dlx5 is associated with proliferation and increased oncogenic potential of tumourigenic cells in which it is expressed (Tan et al. 2010; Xu & Testa 2009; Kato et al. 2008). The direct transcriptional activation of the common oncogene and important cell cycle regulator c-MYC by DLX5 was determined to be a key element in the promotion of proliferation in multiple cell types (Xu & Testa 2009); because of this evidence that Dlx5 is able to directly affect proliferation and cell cycle regulators in the absence of cellular differentiation, we wanted to investigate whether an effect on cell cycle is also a physiological role of Dlx5 during development. Due to the functional redundancy of paralogous Dlx gene pairs, we chose to
include Dlx6 in our study, as the functional equivalency of Dlx genes is an ongoing topic of interest and investigation in our lab.

4.1 Reduction of cell proliferation by Dlx5 and Dlx6 is context-dependent

In this study, the ability of Dlx5 and Dlx6 to reduce cell proliferation in a variety of cell types was established. In C2C12 cells, which undergo Dlx5-mediated differentiation, there was a 20-30% reduction in the growth of cell populations transfected with Dlx5 or Dlx6, even in the absence of detectable expression of either protein at the time of seeding. The loss of plasmid-based expression over the course of selection could in part be explained by the nature of the proteins being expressed. Since the presence of Dlx5 and Dlx6 negatively affects cell proliferation, sustained growth of transfected cells under selection would give a growth advantage to cells that contain the plasmid and therefore express resistance to neomycin but for an unknown reason have reduced Dlx expression. The fact that a negative growth effect is still seen in transfected C2C12 cells after Dlx plasmid expression is lost suggests that Dlx5 and Dlx6 alter these cells in a permanent, rather than in a transient way. Since differentiation is essentially an irreversible change that causes cells to exit the cell cycle, it will be important to determine whether these C2C12 cells are differentiating during the selection period in response to Dlx5 or Dlx6. To do this, detection of markers of differentiation at the time of seeding the growth assay is necessary and is currently under investigation.

In the primary limb bud cells changes in cell proliferation in response to Dlx expression was measured without selection for the transfected cell population. Due to the low transfection efficiency of primary limb bud transfections, and the inconsistent protein expression detected via immunoblot analysis, the modest reduction in cell proliferation seen in Dlx5- and Dlx6-transfected primary limb bud populations is likely an underestimate of the effect of Dlx protein
expression on individual cells. Although Dlx5 and Dlx6 do enhance differentiation of PLB cells, it is unlikely that these cells have differentiated 24 hours post transfection at the relatively low seeding density we used since Dlx5 and Dlx6 are unable to stimulate chondrogenic differentiation at low cell densities (Hsu et al. 2006). Therefore, the decreased proliferation in the Dlx5- and Dlx6-transfected populations is likely due to a direct effect on cell cycle, and not because the cells have differentiated.

The use of avian DF-1 cells allowed us to take advantage of the RCASBP retroviral system. RCASBP(A) vectors encoding Dlx protein are integrated into the DF-1 DNA by reverse transcriptase activity and the cell’s own machinery is used for viral propagation. Any untransfected cells are infected with the RCAS(A)-containing virus and so by the time the growth assay is seeded, almost all the cells should be expressing the transgene and no selection is needed. Protein expression at time of seeding was high, and accordingly, the effect on cell growth was greater than in C2C12 or primary limb bud cells. Expression of RCAS(A)-myc2-Dlx5HDM, a Dlx5 variant that is unable to bind to DNA, did not alter population growth in DF-1 cells. This provides evidence that the transcriptional function of Dlx proteins is required in order to affect cell proliferation.

To the best of our knowledge, DF-1 cells do not undergo differentiation in response to Dlx5 or Dlx6 expression, and no obvious phenotypic changes have been observed in Dlx5- and Dlx6-expressing cells compared to vector control cells (data not shown). The DF-1 cell line is a spontaneously immortalized chick embryonic fibroblast (CEF) cell line, collected from 10-day old embryos. Little information is available on the differentiative potential of DF-1 cells, however, in general, embryonic-derived fibroblast cell lines are multipotent and can be induced to differentiate into various types of muscle, bone, skin and adipose cells. It is likely that forced
expression of \textit{Dlx5} or \textit{Dlx6} would promote differentiation into a skeletal lineage, as it does when overexpressed in primary limb bud and C2C12 cells; however, differentiation markers from multiple lineages should be used if the differentiation potential of DF-1 cells is to be tested in the future. Knowing whether the negative effect on cell proliferation is linked to differentiation would be valuable in the elucidation of the dual role Dlx proteins may play in these processes. The only cell line tested that did not have a positive or negative growth response to Dlx protein expression was the HEK293T cell line. This is interesting, given the results of the transcription assays, which will be discussed in section 4.3.

\textbf{4.2 Dlx5 and Dlx6 decrease the proportion of actively cycling cells in a population}

The cumulative labeling method is a simple way of estimating the total length of the cell cycle in a cell population, as well as the proportion of actively dividing cells. Our results have demonstrated that \textit{Dlx5} or \textit{Dlx6} expression in a cell population decreases the proportion of actively cycling cells. While the slopes of the initial linear increase were not determined to be statistically different in the Dlx+ populations compared to the surrounding Dlx- populations, the lack of statistical significance in our experiment may be due, in part, to experimental design and significant variability between experimental replicates. Because a decreased GF cannot fully account for the increased length of time required to reach the maximum labeling index in the Dlx+ cells, further experimentation may be needed to rule out a possible increase in cell cycle length in \textit{Dlx}-expressing cells. Additionally, experiments in human and mouse embryonic stem cells, as well as in mouse neural stem cells have provided evidence that a lengthening of the G\textsubscript{1} phase of the cell cycle, prior to cell cycle exit, may actually be an early promoter of differentiation, rather than being a consequence of differentiation (Roccio et al. 2013; Filipczyk et al. 2007). While we cannot make any conclusions as of yet, it is possible, that the decreased
growth fraction in Dlx-expressing populations is due to a cell cycle exit, preceded by an increase in the length of the cell cycle. Although our preliminary results suggest an increase in the proportion of cells in G1 in Dlx-expressing populations, our results could also be indicative of increased G1 length. To distinguish between these possibilities, further flow cytometric analysis is needed. The ability to draw conclusions from our current results is limited by the low transfection efficiency we have experienced, the inability to distinguish Dlx-expressing cells from the non-expressing population, and the loss of Dlx expression after selection. To address these problems, we plan on infecting DF-1 cells with Dlx-encoding RCAS viruses, an approach which has previously resulted in an infection efficiency of close to 100% within 24-48 hours (data not shown).

In order to gain insight into the cause-and-effect relationship between cell cycle changes and differentiation, it is important to establish the timing of expression of differentiation markers in relation to cell cycle lengthening and exit. We plan on exploring the presence or absence of markers of osteogenic differentiation, such as alkaline phosphatase, osteocalcin and Runx2, as the upregulation of these genes in C2C12 cells within the first 24 hours of BMP2-stimulated osteoblastic differentiation has been previously established (Balint et al. 2003)

4.3 Dlx5 and Dlx6 promote the transcriptional activation of the proximal c-MYC promoter

In agreement with previously published data, Dlx5 and Dlx6 activated the transcription of a c-MYC reporter in transfected HeLa cells (Xu & Testa 2009). In a more physiologically relevant context, we showed that the c-MYC reporter is upregulated in a dose-dependent manner in Dlx6- transfected C2C12 and primary limb bud cells, which undergo osteogenic or chondrogenic differentiation respectively in response Dlx5 or Dlx6 expression. However, in contrast to Xu and Testa’s findings, which indicate that DLX5-mediated transcriptional
activation of \textit{c-MYC} promotes cellular proliferation, the ability of Dlx5 and Dlx6 to transcriptionally activate the proximal \textit{c-MYC} promoter in C2C12 and primary limb bud cells, as demonstrated with the dual-luciferase transcription assays, did not correlate to an increase in cellular proliferation when Dlx5 or Dlx6 was overexpressed in these cell types.

\textit{c-Myc} is an interesting gene because it is involved in so many different cellular processes including proliferation, differentiation and apoptosis (Zinin et al. 2014; Pelengaris, Rudolph, & Littlewood 2000). Although it is a common oncogene, stimulating proliferation and increasing tumourigenicity of a variety of cancer cell types, the effect of \textit{c-Myc} misexpression on cellular behaviour depends on the environmental context of expression, the presence of other transcriptional regulators, and the amount of c-Myc present (Eilers & Eisenman 2008; Lin et al. 2012). For example, whereas \textit{c-MYC} overexpression in tumourigenic T cells leads to increased proliferation (Xu & Testa 2009), overexpression of \textit{c-Myc} in chick limb buds during development causes an increase in limb size not from an increase in proliferation, but from an enlargement of each individual cell (Piedra et al. 2002). A key to understanding the diverse roles of c-Myc was discovered in 2012, when Lin et al. discovered that rather than activating or repressing specific target genes when expressed, c-Myc binds to the promoters and enhancers of active genes to amplify their expression. At low levels, c-Myc likely binds to the target sequences with which it has high affinity, and as those sequences become saturated, c-Myc would bind to lower affinity target sequences. This would explain both the diverse roles of c-Myc in different cellular contexts and the dose-dependent response of a cell to increasing levels of c-Myc (Lin et al. 2012).

Whether the activation of \textit{c-Myc} is an important part of the physiological roles of Dlx5 and Dlx6 during development is unclear, as the transcription assays are performed in an artificial
in vitro environment, where Dlx proteins are expressed at higher than physiological levels and only part of the c-MYC promoter is used in the reporter system. In vivo misexpression of Dlx5 or c-Myc in chick limb buds both lead to shortened skeletal elements, however the former is due to an acceleration of chondrocyte maturation and a decrease in proliferation, while the latter is due to delayed chondrocyte differentiation (Ferrari et al. 1995; Pera & Kessel 1999; Brown, Wang & Groves 2005; Stock et al. 1996). However, because the context and level of expression of c-Myc is a crucial factor in its physiological role, we cannot rule out that Dlx-mediated activation of c-Myc is an important physiological role of Dlx5 and Dlx6 during skeletal development.

A dose-responsive repression of c-MYC is seen in HEK293T cells, contrary to the activation of this reporter demonstrated in a previous study in this cell line (Xu & Testa 2009). Context dependent action of Dlx proteins on the transcription of a target gene has been shown previously, with conflicting data on the activation or repression of the Osc gene by Dlx5. In osteoblast sarcoma cells and primary rat osteoblasts, Dlx5 is able to repress Osc promoter activity, as measured with a transcription assay, while in C2C12 cells Dlx5 has no effect on this gene (Ryoo et al. 1997). In contrast to this repressive action, forced expression of Dlx5 stimulated the production of endogenous Osc protein in a mouse osteoblast cell line. Moreover, osteoblasts collected from Dlx5(-/-) mice exhibited a reduction in osteocalcin expression compared to wildtype mice (Miyama et al. 1999; Samee et al. 2008). The repression of c-MYC by Dlx5 and Dlx6 in HEK293T cells is likely due to the presence of a co-factor or post-translational modification of Dlx5 and Dlx6 that change their transcriptional activity. Because the Dlx5HD\textsuperscript{M} protein, which should not bind to DNA due to the 3 alanine substitutions in the homeodomain, also repressed c-MYC promoter activity, it is likely that Dlx5 and Dlx6 act through protein-protein interactions with a cofactor to repress this particular promoter in this cell
type. It is interesting that HEK293T cells were the only cells tested whose growth was unaffected by Dlx protein expression. This could mean that transcriptional activation of c-MYC by Dlx5 and Dlx6 is linked to their ability to affect cell proliferation.

We would like to determine other possible transcriptional targets of Dlx5 and Dlx6 that could account for their effects on cell growth. To do this, we will perform quantitative reverse-transcriptase polymerase chain reaction (qPCR) using primers for various cyclins and CKIs to detect differences in detection levels in Dlx5/6-expressing populations compared to vector control populations. The promoters of cell cycle regulator that exhibits altered expression levels in the presence of Dlx proteins will be examined, in order to determine the presence of Dlx binding sites (TAATT). Using transcription assays, we will establish the ability of Dlx5 and Dlx6 to affect the transcriptional activation of these genes.

4.4 Dlx5 and Dlx6 are functionally equivalent in the control of cell proliferation

Of ongoing interest in our laboratory is the functional comparison of all 6 Dlx homeodomain proteins. Dlx5 and Dlx6 are cis-linked paralogues that show functional overlap, even though the encoded proteins are quite dissimilar in their amino and carboxyl-terminal domains (Hsu et al. 2006). The phenotypic changes seen in Dlx5 or Dlx6 single knockouts are less severe than in Dlx5/Dlx6 double knockouts and Dlx5 can rescue defects in chondrocyte differentiation in Dlx5/Dlx6 knockout mice (Depew et al. 2005; Zhu & Bendall 2009). Results of our growth curve and EdU cumulative labeling experiments indicate that in the control of cell proliferation, Dlx5 and Dlx6 are also functionally equivalent.
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Figure A.1 Analysis of Dlx5 cumulative EdU labeling assay

(A) All data points represent 3 experiments, except the 16 hour incubation time, which represents 2 experiments. In order to determine the maximum EdU labeling index (GF), a series of linear regressions were performed, starting with the last two points and incorporating earlier incubation times until the regression lines were significantly different from 0. For both Dlx5- and Dlx5+ populations, including the 8 hour incubation time significantly altered the regression lines and therefore were not included when determining the GF. (B) Slopes of the initial linear increase were therefore determined by using all points with a lower LI than the maximum. Because of this, the 16 hour incubation time point was included in both the maximum and the initial linear increase. (C) Y intercepts of the regression lines for the GF points were compared and determined to be significantly different from each other (**p<0.0001). (D) The maximum labeling index was determined by applying a horizontal, least squares ordinary fit to the GF points.
Figure A.2 Analysis of Dlx6 cumulative EdU labeling assay

(A) All data points represent 3 experiments, except the 16 hour incubation time, which represents 2 experiments. In order to determine the maximum EdU labeling index (GF), a series of linear regressions were performed, starting with the last two points and incorporating earlier incubation times until the regression lines were significantly different from 0. For both Dlx6- and Dlx6+ populations, including the 8 hour incubation time significantly altered the regression lines and therefore were not included when determining the GF. (B) Slopes of the initial linear increase were therefore determined by using all points with a lower LI than the maximum. Because of this, the 16 hour incubation time point was included in both the maximum and the initial linear increase. (C) Y intercepts of the regression lines for the GF points were compared and determined to be significantly different from each other (**p<0.0001). (D) The maximum labeling index was determined by applying a horizontal, least squares ordinary fit to the GF points.
ACTGCTACGG AGGAGCAGCA GAGAAAGGGA GAGGGTTTGA GAGGGAGCAA AGAAAAATGG
TAGGCAGCGG TAGCTTAATT GATGCAGGTCT CTTCACCGCT GTATAGTCCAT GAGCTAGAGT
GCTCGGCTGC CCGGCTGAGT CTCTCTCCCA CCTCCCCCAC CTCCCCATTA
CGCCCCCTCC GGGTTCCCA AAGCAGAGGG CTGGGGGAAA AAGAAAAAG ATCCCTCTCTC
GCTAATCTCC GCCCCACCGGC CCTTTATAAT GCGAGGCTCT GAGCGGCTGA GGACCCCGGA
GCTGTGCTGC TCAGGCGCCGC CACCCGCCGG CCCCGGCCGT CCTGCCCTTC CTCCTCTCTC
CGAGAAAGGC AGGGCTTTCG AGAGGCTTGG CCGGAAAAAG AAGGGAGGG GGGATCGCGC
TGAGTATAAA AGCCGGGTCTT TCTAACTCGC TGTAGTAATT CCAGCGAGAG
GCAGAGGGAG CGAGCAGGCG GCC

Figure A.3: The proximal c-MYC promoter contains two Dlx binding sites
High-affinity Dlx5 and Dlx6 binding sites are highlighted. This portion of the c-MYC promoter was cloned into a pGL2 backbone by Facchini et al. (1997).
Figure A.4: VDD5 activates c-MYC transcription in HeLa cells

HeLa cells were seeded in triplicate into 12-well dishes at a density of $1.2 \times 10^5$ cells/well and transfected 24-hours later. Cells were collected 24 hours post transfection and resuspended in 40μL of PLB. 10μL of lysed sample was measured per reading. Bars represent fold activation +/- SEM, relative to the vector control, set at 1 (n=2). Dose-responsiveness was tested using ANOVA **p<0.0001. The maximum activation of VDD5 was determined to be significantly different from the basal activation in the vector control using a paired t-test (p<0.05).