NR4A1, NR4A2, and NR4A3 are three members of an orphan nuclear hormone receptor family referred to as Nur77 family. NR4A1 is also called Nur77, TR3, or NGFI-B; NR4A2 is also named as Nurrl, RNR-1 or TONOR; while NR4A3 has also been called Minor and Nor-1. They are all transcription factors belonging to the superfamily of steroid nuclear hormone receptors (Forman et al., 1995; Mangelsdorf et al., 1995; Enmark and Gustafsson, 1996). The structures of these orphan receptor proteins have been reviewed in detail elsewhere (Hsu et al., 2004); they include an activation domain at their N-terminus (A/B or A/F-1), a DNA binding domain (C or DBD) (Wilson et al., 1991) and a ligand binding domain (E, LBD, or AF-2) at their C-terminus (Enmark and Gustafsson, 1996) (Fig. 1, Table 1). NR4A1, 2 and 3 are highly homologous in the DBD domain (~91-95%), modestly homologous in the LBD domain (~60%), and very divergent in their activation domain (Table 1). All three members are found in the nucleus since their DBDs also contain nuclear localization signals (NLS) (Katagiri et al., 2000). The LBD of NR4A1 contains three nuclear export signals (3xNESs) as well as a Bcl-2 binding domain (Table 1) (Katagiri et al., 2000; Lin et al., 2004). The DBD’s bind to the consensus DNA sequence AAAGGTCA (NBRE, NGFI-B response element) as monomers, and to AAAT(G/A)(C/T)CA (NurRE, Nur-responsive element) by homodimerization or heterodimer interactions with each other to activate different target gene expression (Perlmann and Jansson, 1995; Zetterstrom et al., 1996; Maira et al., 1999). Dimers show stronger activity over monomers, and different dimers exhibit different preferences for targeted DNA sequences. Nur77 family proteins are involved in a wide variety of biological processes, especially the ones involved in cell survival and apoptosis. They are immediate early genes induced by serum, growth factors and receptor engagement (Winoto and Littman, 2002).
Crystal structures of the functional domain of the members have been described, e.g. the DNA binding domain of NR4A1 complexed with its target DNA (Meinke and Sigler, 1999), and the LBD of NR4A2 and NR4A4. NR4A4 being the Drosophila ortholog of NR4A1, 2 and 3 (Baker et al., 2003; Wang et al., 2003). The crystal structure of the LBD of NR4A2 and their Drosophila ortholog NR4A2 are similar to the agonist-bound, transcriptionally active LBDs of other nuclear hormone receptors. The “classical” ligand-binding pocket on LBD is tightly packed with side chains from several conserved bulky hydrophobic residues (Wang et al., 2003). NR4A2 lacks the classical binding site for co-activators. However, the LBD of NR4A2 can still be regulated in mammalian cells, and its transcriptional activity is correlated with the NR4A2 LBD adopting a more stable conformation. Unlike other steroid receptors, which are constitutively expressed and their activity modulated by steroid ligands, the expression of NR4A1, 2 and 3 appears to be regulated by external stimuli through their expression. The regulation of NR4A1 transcription has been extensively reviewed (Zetterstrom et al., 1996; Winoto and Littman, 2002; Hsu et al., 2004).

Although these receptor transcription factors are considered to be orphan, they can be regulated by many hormones via the formation of heterodimers with other hormone receptors that do bind hormone ligands. These receptors can also influence the target genes of other nuclear receptors via formation of similar heterodimers. Furthermore, a recent study revealed that NR4A1 can directly bind to synthetic ligands and thereby modulate its activities (see below, (Chintharlapalli et al., 2005)).

**NR4A1/2/3 receptor family in cell survival and apoptosis**

Expression of these three receptors in tissues and organs varies, which may imply diverse physiological functions (Table 2). NR4A1 is widely expressed, but primarily in thymus, osteoblast, liver, and pituitary gland (Zetterstrom et al., 1996; Hsu et al., 2004). NR4A2 is expressed in the developing and adult central nervous system (Law et al., 1992; Paulsen et al., 1995; Le et al., 1999). NR4A3 mRNA was detected at low levels in the adult heart and skeletal muscle as well as in the fetal brain. It is expressed at high levels in the pituitary gland. The expression kinetics of NR4A3 is similar to that of NR4A1 in activated T cells, and they appear to play partially redundant roles in T cells and adrenal glands (Cheng et al., 1997; Fernandez et al., 2000). This putative redundancy may explain the complete lack of phenotype in NR4A1 knockout animals. Transgenic mice (Calnan et al., 1995; Weih et al., 1996) and knockout mice (Cheng et al., 1997; Zetterstrom et al., 1997; Castillo et al., 1998; DeYoung et al., 2003; Hsu et al., 2004) have also been used to investigate the roles of these receptors in vivo. The phenotypes observed in some transgenic and knockout animal models are summarized in Table 3.

NR4A1, 2, and 3 have all been previously implicated in cell growth/survival and apoptosis. These functions appear to be derived from three biochemical properties of these receptors: 1) as transcription factors whose activation up-regulates target gene expression leading to cell proliferation and survival (Bras et al., 2000; Suzuki et al., 2003; Ke et al., 2004); 2) as transcription factors whose activation up-regulates target genes leading to cell apoptosis (Zhang et al., 1999; Chintharlapalli et al., 2005); 3) translocation to the cytoplasm and targeting mitochondria, thus triggering apoptosis (Li et al., 2000; Lin et al., 2004). These pro-survival and anti-survival biological functions in cells have been shown to play important physiological as well as pathological roles.

A variety of experimental tools have been used to study the biological functions and biochemical properties of the Nurr1 family of receptors. Over-expression (gain-of-function) (Bras et al., 2000; Suzuki et al., 2003), dominant negative mutants (Suzuki et al., 2003), antisense (Zhang et al., 1999) and more importantly, the recent RNAi-mediated gene-silencing (loss-of-function) (Kolluri et al., 2003; Ke et al., 2004; Chintharlapalli et al., 2005; Li et al., 2005) technologies have been broadly used to study the in vitro functions of these receptors. Since these receptors are transcription factors that share recognition sequences, these sequences can be used to generate reporter systems to investigate their activity. The downstream gene transcription activities also provide ways to explore the biological functions of these receptor molecules. Several types of

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**Table 1. Summary of the functional domains of Nur77 family.**

<table>
<thead>
<tr>
<th>DOMAIN</th>
<th>DESIGNATION</th>
<th>CONSERVATION</th>
<th>FUNCTION</th>
</tr>
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<tbody>
<tr>
<td>A/B</td>
<td>AF-1</td>
<td>Low (21-27%)</td>
<td>Transactivation</td>
</tr>
<tr>
<td>C</td>
<td>DBD</td>
<td>High (&gt; 90%)</td>
<td>zinc finger motif, DNA binding, 2x NLSs</td>
</tr>
<tr>
<td>D</td>
<td>Variable hinge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>LBD, AF-2</td>
<td>Intermediate (~ 60%)</td>
<td>Transactivation, 3x NESs, Bcl-2 binding</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>unknown</td>
<td></td>
</tr>
</tbody>
</table>

AF-1: Activation function-1C-domain function; DBD: ligand binding domain, dimerization domain, region, nuclear localization domain; AF-1: activation function-1; DBD: DNA binding domain contain NLSs (nuclear localization signals); NESs: nuclear export signals for NR4A1.
reporter systems have been shown to be extremely useful in exploring the biochemical and biological roles of these receptors. These includes:
1) NurRE-luciferase (or other reporter) can be used to assess transactivation activity (Ke et al., unpublished observations) (Fig. 1);
2) GAL4-NR4A1 (LBD)-luciferase can be used for screening functional binders for NR4A1 (Chintharlapalli et al., 2005) (Fig. 1);
3) GFP-TR3 fusion protein can be used for TR3 localization/translocation upon treatment (Lin et al., 2004) (Fig. 1).

**NR4A1**

**Pro-survival role of NR4A1**

NR4A1 is the most well studied member among this receptor family. Its expression can be induced by a number of different stimuli, e.g. stress stimuli like TNF (Suzuki et al., 2003), serum, and growth factors including NGF and T-cell receptor mediated signaling. Over-expression of NR4A1 was shown to prevent ceramide-induced cell death in a mature B-cell lymphoma (A20) (Bras et al., 2000), suggesting a pro-survival or anti-apoptotic function for NR4A1. However, this over-expression did not antagonize FasL induced apoptosis in the same cells, suggesting that the observed anti-apoptotic function does not involve Fas-mediated apoptosis pathway. Others have also reported that over-expression of NR4A1 in certain human lung cancer cells caused resistance to retinoic acid treatment and contributed to proliferation and neoplastic transformation (Wu et al., 1997).

In a subsequent study, the ectopic expression of NR4A1 in mouse embryonic fibroblasts (MEF) was shown to antagonize TNF induced apoptosis while a negative dominant mutant defective in the transactivation domain accelerates TNF induced cell death (Suzuki et al., 2003). This once again indicates that NR4A1 is a survival effector in the TNF survival pathway. Furthermore, both caspase 3 and caspase 8 activities were reduced in cells over-expressing NR4A1, indicating the involvement of these two apoptosis enzymes. Additionally, NR4A1 was localized to the nucleus throughout these observations, indicating that its role as a transcription factor probably contributes to this effect.

NR4A1 silencing mediated by siRNA leads to a drastic reduction in cell growth/survival, as we have demonstrated in many cancer cell lines (Table 4). The reduced growth correlated well with increased levels of apoptosis. For example, transient transduction of cancer cells with lentiviral RNAi vector caused apoptosis (Ke et al., 2004) (Table 4). Consistent with our observation, the mitogenic function of NR4A1 is also observed in lung cancer cells (Kolluri et al., 2003). Therefore, the pro-

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**Table 2. Normal tissue expression patterns of NR4A1,2,3.**

<table>
<thead>
<tr>
<th></th>
<th>NR4A1</th>
<th>NR4A2</th>
<th>NR4A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brain</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Osteoblast</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Adult heart</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Domains structures and reporter assays. A. Domain structure for NR4A1 family protein is shown. B. NurRE-luciferase. Nur77-response element (NurRE) is cloned in front the TK-luciferase construct. Binding of NR4A1/2/3 will activate reporter expression. C, D. Gal4/GRE-luciferase and Gal4/DBD-NR4A1 (C)/LBD (D). The reporter is constructed by cloning the GAL response elements (GRE) in front of the TK-luciferase constructs. GAL4 DNA binding domain fused either with full-length Nur77 (C) or Nur77 ligand binding domain (D). Binding of the GAL4-Nur77 or GAL4-Nur77/LBD will activate the reporter expression. E. GFP-TR3.
survival function of NR4A1 has been demonstrated by both gene over-expression and silencing studies for both the extrinsic and intrinsic apoptotic pathways.

**Pro-apoptotic role of NR4A1**

Interestingly, NR4A1 can also mediate an opposite biological effect, namely a pro-apoptotic effect. NR4A1 was reported to be pro-apoptotic in thymocytes (Zhang et al., 1999; Winoto and Littman, 2002). In self-reactive immature thymocytes, NR4A1 expression was induced and increased apoptosis was observed following stimulation of the T cell receptor (TCR). Expression of a dominant negative NR4A1 mutant or antisense blocked the TCR dependent apoptosis. Over-expression of NR4A1 in transgenic mice resulted in high levels of apoptosis in thymocytes (Calnan et al., 1995), confirming its pro-apoptotic role in vivo and thus its physiological relevance. Interestingly, when knockout animals were generated, no phenotype was observed (Lee et al., 1995), suggesting the likely redundancy of gene functions, e.g. with NR4A3 (see below). It is worth noting that the above described opposing pro-apoptotic and anti-apoptotic effects can even occur within the same cell system, e.g. NCI-H460 (Li et al., 1998; Kolluri et al., 2003) depending on the context (e.g. specific apoptotic stimuli) and the localization of NR4A1. However, the exact regulation of the two opposing effects is still to be elucidated.

The pro-apoptosis role of NR4A1 may be achieved by the transactivation function of NR4A1 as a transcription factor by targeting genes responsible for promoting apoptosis, e.g. FasL or TRAIL (Zhang et al., 1999; Chintharlapalli et al., 2005). Thus, this function is associated with nuclear localization of NR4A1. However, studies also revealed a unique mechanism in which NR4A1 translocates to the mitochondria and binds to Bcl-2 to form a pro-apoptosis complex in response to apoptotic stimuli. This triggers cytochrome C release and apoptosis as demonstrated in LNCaP human prostate and other cancer cells (Li et al., 2000). Domain analysis indicated that the LBD is responsible for Bcl-2 binding. This binding results in the conformational change of Bcl-2, which subsequently leads to NR4A1 mitochondrial localization (Lin et al., 2004), although NR4A1 lacks classic mitochondria-targeting sequences. The presence of three putative nuclear export sequences (NESs) in the LBD is probably responsible for the translocation out of the nucleus. These events cause activation of Apaf-1 and caspase 9 followed by cleavage of caspase 3 and apoptosis.

Although the mechanisms of regulation of NR4A1 activation is not completely understood, the phosphorylation status of NR4A1 appears to play an important role in its transcriptional activity. Phosphorylation, which can be carried out by Akt or the MAPK family kinases, results in predominantly cytoplasmic localization of the protein (Hsu et al., 2004). The extent of phosphorylation depends on the nature of the stimuli, resulting in differential regulation of its transcriptional activity. Nerve growth factor (NGF) probably induces NR4A1 expression and phosphorylation at Ser350 in the DBD, which impairs its binding to NBF (Hirata et al., 1991; Katagiri et al., 1997). Akt kinase is also shown to target Ser316 in a PI-3K-dependent manner, thus inhibiting DNA binding and gene activation, resulting in suppression of NGFI-B-induced apoptosis in fibroblasts and TCR-mediated cell death in T-cell hybridomas (Masuyama et al., 2001; Pekarsky et al., 2001). NGF also induces additional phosphorylation at Ser105 through the Trk/Ras/MAPK pathway, resulting in nuclear export of NR4A1 (Katagiri et al., 2000) along with RXR via heterodimerization. In contrast, dephosphorylation of Ser316 through adrenocorticotropic (ACTH) treatment in adrenal-derived Y1 cells allows DNA binding of NR4A1 (Davis and Lau, 1994; Li and Lau, 1997).

**NR4A2**

In contrast to NR4A1, only pro-survival effects, but not pro-apoptotic effects, have been reported for NR4A2. NR4A2 is highly expressed in brain tissues (Table 2), with lower expression in some several other tissues. NR4A2 knockout mice were impaired in development and maintenance of midbrain dopaminergic neurons, and they die shortly after birth (Law et al., 1992; Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998). Heterozygous mice (NR4A2 +/-) appear healthy, but were more susceptible to the action of neurotoxins (Le et al., 1999). NR4A2 has also been linked to Parkinson’s disease, as mutations in

<table>
<thead>
<tr>
<th>ORPHAN RECEPTORS</th>
<th>TRANSGENIC PHENOTYPES</th>
<th>KNOCKOUT PHENOTYPES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR4A1</td>
<td>Massive thymocyte apoptosis</td>
<td>Minimal phenotype</td>
<td>(Lee et al., 1995; Cheng et al., 1997)</td>
</tr>
<tr>
<td>NR4A2</td>
<td>NT</td>
<td>Development lethal (die right after birth); lack dopamine-producing neuron</td>
<td>(Zetterstrom et al., 1997)</td>
</tr>
<tr>
<td>NR4A3</td>
<td>NT</td>
<td>Embryonic lethal; defect in proliferation of the semicircular canals of the mouse inner ear</td>
<td>(Ponniio et al., 2002; DeYoung et al., 2003)</td>
</tr>
</tbody>
</table>

NT: not tested.
the NR4A2 gene were recently found in certain patients (Le et al., 2003). Over-expression of NR4A2 increased the resistance of neural stem cells to neurotoxins 6-hydroxy-DA and MPTP (Lee et al., 2002). Together, these findings point towards a cyto-protective function for NR4A2 in the CNS.

Pro-survival/anti-apoptotic properties have also been observed for NR4A2 using a transient siRNA transduction approach and a lentiviral NR4A2-siRNA vector in several other cancer cell lines of different origin, similar to that of NR4A1. In these experiments, silencing of NR4A2 caused apoptosis assessed by TUNNEL-based assays (Table 3) (Ke et al., 2004). However, stable NR4A2-silenced cancer cells can be generated for HeLa cells, PC3 and HCT116 cells and these stably transduced cells displayed greatly reduced anchorage-independent growth, with minimal reduction in anchorage-dependent growth, as measured by cloning efficiency assays. Further experiments indicate that this reduced anchorage-independent growth was largely due to increased anoikis (apoptosis caused by cell detachment). Since anchorage-independent growth is a hallmark of cell transformation, this result strongly suggests a role for NR4A2 in cell transformation. This was further confirmed by the failure of cancer cells transduced with the NR4A2 siRNA vector to establish tumor in the xenograft model (Ke et al, unpublished).

NR4A3

NR4A3 has been shown to be functionally redundant with NR4A1 in T-cell apoptosis (Cheng et al., 1997). The tissue expression pattern of NR4A3 is also similar to that of NR4A1 (Cheng et al., 1997) (Table 2). On the other hand, our unpublished data also indicate a pro-survival role of NR4A3 (Ke and Sundaram et al., unpublished observations). Our observations are further supported by other studies in which NR4A3 knockout mice were shown to be embryonic lethal (DeYoung et al., 2003), similar to NR4A2 knockout mice, but in contrast to NR4A1 knockout mice (no phenotype). These results also suggest that NR4A1 and NR4A3 have non-overlapping functions as well.

In summary, all three members of Nurr77 receptor family seem to have pro-survival function in certain contexts, while NR4A1 and NR4A3 also have pro-apoptotic functions in other contexts.

NR4A1/2/3 and potential roles in human cancers

Earlier reports have described the possible involvement of NR4A1, 2, and 3 in a variety of human diseases (Le et al., 2003; Hsu et al., 2004), including NR4A1 in inflammatory disease, allergy and atherosclerosis, and NR4A2 in Parkinson disease and rheumatoid arthritis (McEvoy et al., 2002). However, the accumulated data also implicate this family of receptors in cell transformation and human carcinogenesis. A strong evidence for the role of the Nur77/NGFIB subfamily of nuclear hormone receptors in cell transformation is the observation that all three members are down-regulated in non-transformed HeLa/HF revertant cells as compared to the transformed parental HeLa cells (Fig. 2) (Boylan et al., 1996; Ke et al., 2004; Li et al., 2005). HeLa is a cervical cancer cell line exhibiting typical transformed phenotypes, including anchorage-independent growth in vitro and tumorigenesis in vivo. HeLa/HF is a revertant variant isolated from HeLa cells following exposure to the mutagen ethane methyl sulfonate. Interestingly, when HF cells were re-transformed and became tumorigenic, all members of this family were also up-regulated. Conversely, silencing of these genes in HeLa cells caused a reversal of cell transformation (see above), confirming the causal roles of these genes in cell transformation. Our recent unpublished data that the silencing of NR4A2 causes a drastic reduction in tumorigenicity of PC3 prostate cancer model further underscores the importance of this receptor for cell transformation and tumorigenicity (Ke et al., unpublished).

The second evidence that NR4A1 is involved in cancer came from the observation that a 270 fold-enhanced transcription of NR4A1 occurs in extraskeletal contexts.
myxoid chondrosarcoma due to a chromosomal translocation (Labelle et al., 1999). There is also a paradoxical association of high levels of Bcl-2 with favorable clinical outcome for patients with several types of cancers, including breast, colon, and NSCL cancers. The observation that NR4A1 binds Bcl-2 may provide a possible mechanism for the above observation, since NR4A1 binding to Bcl-2 induces apoptosis (Lin et al., 2004).

NR4A1/2-3 as potential drug targets for human diseases

The cumulative information suggests that the Nur77/NGFIB family of nuclear hormone receptors may share similar pro-survival functions at least in certain cancer cells, and thus these properties could potentially be explored to develop antagonist drugs for cancer. Since RNAi mimics antagonist activity, our in vivo RNAi experiment with NR4A2 suggests that this receptor can serve as a drug target provided that antagonists can be identified. On the other hand, the pro-apoptosis nature of NR4A1 can also be explored to develop agonist therapeutics (Chintharlapalli et al., 2005).

The absence of a conventional ligand-binding pocket raises uncertainty for identifying modulators for the functions of these receptors. However, a recent study identified a novel coactivator interaction site between helices 11 and 12 for both NR4A2 and NR4A1, instead of the classical coactivator interaction site in the E/F domain (helix 12) (Codina et al., 2004). In addition, Chintarlapalli and colleagues identified an NR4A1-binding agonist, which demonstrates the possibility of activity-modulators for this family of receptors (Chintarlapalli et al., 2005). This study described that 1,1-bis (3'-indolyl)-1-(p-substituted phenyl) methane-containing trifluoromethyl, hydroxyl and methoxy substituents (also called selected C-substituted DIMs (3,3'-diindolylmethanes)) induced NR4A1-dependent transactivation in cancer cell lines (e.g. Panc28 pancreatic cancer cell line) and apoptosis. These active compounds were identified by screening based on GAL4-Nur77/GAL4 reporter assay, in which a structure-dependent activation of NR4A1 was monitored in cells co-transfected with GAL4-Nur77 (full-length) chimera, or GAL4-Nur77 (E/F)(LBD or E/F domain) chimera, along with a reporter vector containing five GAL4 response elements linked to luciferase gene (Fig. 1C, D).

In summary, it is clear that NR4A1, NR4A2 and NR4A3 orphan receptors play different roles in cell transformation. These properties can potentially be utilized as targeted anti-cancer therapy. A new class of antagonists against NR4A1 has been identified for this utility, which can serve as a paradigm for the search of antagonists for other members of this unique receptor family.

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NR4A1, 2, 3 orphan receptor family and apoptosis

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