Chapter 4

*DRD4* expression in human lung epithelial cell lines: A novel finding
**DRD4 expression in human lung epithelial cell lines: A novel finding**

**4.1 Introduction and rationale of the study:**

Dopamine receptors have been identified in a number of organs and tissues, which include the central and peripheral nervous systems, the gastrointestinal tract, kidney, various vascular beds, and the heart. Dopamine does not cross the blood-brain barrier and dopaminergic signalling in brain is functionally distinct from peripheral pathways. So, where does dopamine in peripheral blood and tissues arise from?

Three main peripheral sources for dopamine are reported: i) Sympathetic nerves (neuronal fibres); ii) Adrenal medulla (chromaffin cells); and iii) Neuroendocrine (APUD) cells of kidney (Wahbe et al., 1982; Mappouras et al., 1990), of both exocrine (Mezy et al., 1996) and endocrine (Rubi et al., 2005) of pancreas, of retinal cells (Kubrusly et al., 2008), and of peripheral leukocytes (Kokkinou et al., 2009). It is quite striking that, though there are no specific dopamine sources in peripheral tissues, the concentration of free dopamine in plasma is similar to that of adrenaline. Further, conditions like stress, hypovolemia, exercise etc increase the plasma dopamine levels, suggesting an origin in sympathetic nerves. Just like brain neurons (Lloyd et al., 1970), sympathetic nerves and cells of the adrenal medulla also express L-amino acid decarboxylase, which can synthesize dopamine from L-DOPA (Wahbe et al., 1982). It is believed that release of dopamine from sympathetic nerves, adrenal glands and APUD cells and the nerve-derived dopamine synthesis may have a common regulation. Several actions of peripheral dopamine have been described in literature. Peripheral dopamine via its receptors regulates respiration (Pardal et al., 2007), gastrointestinal motility, and blood pressure (Biaggioni et al., 1987). Notably, dopamine signalling in the retina is essential for light dependent control of circadian rhythms (Doi et al., 2006).

Role of dopamine synthesis and its respective D2 –like receptors (D2, D3 and D4) in regulation of glucose homeostasis and body weight are well explained and accepted through genetic association, pharmacological (antipsychotics) and physiological
DRD4 in lung epithelial cells

studies (Melkersson et al., 2004 & 2007). Through peripheral dopaminergic system, dopamine controls (decreases) the blood pressure via renal and non renal mechanisms. In kidney, all dopamine receptor subtypes participate in the modulation of sodium balance via Na⁺/K⁺ ATPase activity to maintain blood pressure. This hypothesis was supported by in vivo studies where animals lacking any of the dopamine receptors (D1\textsuperscript{-/-}, D2\textsuperscript{-/-}, D3\textsuperscript{-/-}, D4\textsuperscript{-/-}, and D5\textsuperscript{-/-} knockout mice) exhibited high blood pressure (Zeng et al., 2008). In addition to blood pressure modulation, dopamine through its D2-like receptors has been shown to inhibit the secretion of procoagulant von Willebrand factor from human endothelial cells (Zarei et al., 2006). Von Willebrand factor is a well recognized risk factor, which appears in high levels in coronary heart disease (Vischer et al., 2006). By abolishing the phosphorylation (Sinha et al., 2009) of vascular endothelial growth factor (VEGF) receptor, dopamine decreases vascular permeability induced by the cytokine VEGF (Basu et al., 2001). Dopamine receptor ligands (agonist and antagonist) seem to be playing an important role in metabolic changes and its cardiovascular consequences. Treatment with antipsychotics is linked to changes in glucose homeostasis, potentially leading to obesity and type-2 diabetes (Bergman et al., 2005). Atypical antipsychotic clozapine, used in the treatment of schizophrenia increases the risk of thrombotic events, which can occur during the initial days of treatment before the onset of weight gain (Hagg et al., 2002). This antipsychotic treatment may provoke cardiovascular diseases through increased secretion of the procoagulant von Willebrand factor (Zarei et al., 2006) and blockade of dopamine receptors in vascular epithelium (Vischer et al., 2006).

Dopamine receptors also play a crucial role in cell survival, cell proliferation and apoptosis in a tissue specific manner. It promotes cell proliferation and survival in non-transformed cells, whereas in tumor cells it exhibits antiproliferative role. The hypothesis that dopamine could modulate proliferation of pancreatic islet cells emerged when notable transient expression of dopamine-synthesizing enzymes was observed in pancreatic precursors (Hoglinger et al., 2004). Moreover, D2 receptor knockout in mice exhibit lower β-cell mass and replication rate, indicating that D2 receptors play an important role in β-cell mass proliferation in untransformed cells (Tornadu et al., 2010). Apart from the role of promoting cell proliferation, dopamine
might also have a protective role against apoptosis in non cancerous tissues or cells (Nair et al., 2008). In contrast to this effect in differentiated cells, dopamine seems to exert mainly an inhibitory effect on cancer growth.

D2-like receptor agonist bromocriptine (Ishibashi et al., 1994) and SKF-38393, a selective D1/D5 receptor partial agonist, have been shown to inhibit proliferation of human small lung cancer cells and human meningioma cells in culture (Schrell et al., 1990) respectively. These findings along with the additional observation of a mouse model which lacks the dopamine transporter, displays elevated dopamine levels and reduced tumoral growth (Asada et al., 2008) reiterates the important role of dopamine and its receptors in anti-proliferation characteristics. Administration of anticancer drugs for breast and colon tumor along with dopamine increases its efficacy (Sarkar et al., 2008) and this was justified by the observed inhibitory role of dopamine on neovessel formation in tumors (Chakroborty et al., 2008). Mice lacking dopamine D2 receptors manifested adenomas, and few polymorphisms (−141Cdel, T957C and A1412G) of this gene have been shown to be associated with risk of colorectal cancer (Gemignani et al., 2005). Dopamine-related drugs might be envisaged as new targets in the treatment of metabolic syndrome, cardiovascular diseases, diabetes, obesity, and cancer.

As mentioned above, DRD4 has been reported to be expressed in the peripheral system like lymphocytes, heart and kidney and is identified by immunohistochemistry, RT-PCR and ligand binding studies. Role of dopamine receptor expression in peripheral system has been the main focus in cardiovascular research, tumor biology and human renal physiology. Further, stimulation of dopamine receptors with dopamine increases lung edema clearance via alveolar epithelial Na⁺/K⁺-ATPase activity. In lung epithelia, dopamine increases Na⁺/K⁺-ATPase activity and this involves two mechanisms: i) Activation of D1 receptor results in rapid recruitment of Na⁺/K⁺-ATPase molecules from intracellular compartments to the plasma membrane (Ridge et al., 2002); ii) Activation of D2 receptor results in increased transcription of the Na⁺/K⁺-ATPase -1 isoform that results in increased Na⁺/K⁺-ATPase activity in cells that have been exposed to dopamine for 24 hrs (Guerrero et al., 2001). Many studies have demonstrated that,
during bronchial obstruction, inhalation of dopamine produces the bronchodilatory effect in asthma patients (Cabezas et al., 1999 & 2003; Michoud et al., 1984). Further, dopamine D2-like receptor has been identified in the dorsal root ganglia, airway projecting sensory neurons (Peiser et al., 2005 & 2009) and airway smooth muscle cells (Mizuta et al., 2012). Though DRD4 along with other subtypes D1 and D2 receptors are shown to express in the peripheral dopaminergic system, their physiological role is unclear (except angiotensin II-type I receptor mediated hypertension in DRD4 knockout mice (Bek et al., 2006)) and their signalling mechanism is yet to be explored. Studies between dopamine receptor/transporter gene polymorphisms and the possible risk of non-small cell lung cancer have revealed that −521C > T variant in the upstream region of DRD4 is associated with a two- to five-fold increased Non-small-cell lung carcinoma risk. These interesting observations and D2 receptor expressions in lung epithelial, airway smooth muscle cells and its anti-proliferative role in tumors led us to hypothesize that DRD4 (subtype of D2-like receptor) is expressed in lung cell lines. In this study, we provide evidence that DRD4 is expressed in BEAS-2B (transformed human bronchial epithelial cells) and A549 (non small cell lung cancerous cell line), and exhibits classical Gi–coupling adenylyl cyclase activity.

4.2 Materials:

4.2.1 Cell lines:

a) BEAS-2B (adenovirus 12-SV40 hybrid virus transformed, non-tumorigenic human bronchial epithelial cell line) and

b) A549 (adenocarcinoma human alveolar basal epithelial cells) were used in this study.
4.2.2 Cell culture growth medium and related consumables:

Bronchial Epithelial Cell Growth Medium (BEGM) -bullet kit (Lonza Group Pvt ltd, USA), Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life technologies), PBS, Trypsin, T-25 flasks, 6-well plates, cell scrapers.

4.2.3 Chemicals and kits:

Qiagen total RNA isolation kit, MMLV Reverse Transcriptase 1st-Strand cDNA synthesis kit (Epicentre Biotechnologies, Madison, USA), DRD4 antibodies, Paraformaldehyde, Bovine serum albumin, Supersignal west pico chemiluminescent kit, Lance ultra cAMP detection kit, dopamine, forskolin, DRD4 agonist, DRD4 antagonist, spiperone, details of which are provided in chapter 2.
Flowchart of methodology for demonstration of \textit{DRD4} expression and function in human lung cell lines

Lung cell lines (BEAS-2B & A549)

- RNA isolation
- cDNA synthesis and amplification of gene of interest (\textit{DRD4})
- Confirmation of \textit{DRD4} expression

Characterization of \textit{DRD4} expression by Adenylate cyclase inhibition assay

- Total cell lysate
- Confirmation of \textit{DRD4} expression by Western blot and immunofluorescence assay

Further characterization to unravel the role of \textit{DRD4}, not a part of this study
4.3 Methods:

4.3.1 Cell culture:
BEAS-2B cells were grown in BEGM along with the supplementary cocktail provided as per the manufacturer’s suggestions; A549 cells were grown in DMEM with 10% fetal bovine serum. The cells were maintained under normal culture conditions with 5% CO₂ and at 37°C.

4.3.2 Total RNA isolation:
Both BEAS-2B and A549 cells were grown upto ~70% confluence, trypsinized as per routine lab protocol and harvested by centrifugation. Pellet was lysed and the total RNA was isolated using RNeasy Mini Kit (Qiagen) as per manufacturer’s instructions. Total RNA quantity (per µl) and purity was checked using NanoDrop™.

4.3.3 cDNA synthesis and RT-PCR:
cDNA was synthesized from 1µg total RNA using MMLV Reverse Transcriptase 1st-Strand cDNA synthesis kit (Epicentre Biotechnologies, Madison, USA), as per manufacturer’s instructions. Gene-specific primers for DRD4 were used to check the expression levels of DRD4 in both the cell lines. β-actin was used as an internal control.
**Table 4.1 Primer sequences used for the amplification of** **DRD4** **and β-actin**

<table>
<thead>
<tr>
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<th><strong>huDRD4</strong></th>
<th><strong>huβ-actin</strong></th>
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<tbody>
<tr>
<td><strong>FP</strong></td>
<td>5'-GCT CTC CTG GTG CTG CCG CTC TTC GTC TAC T-3’</td>
<td>5'-TCA TGA AGT GTG ACA TGG TAC TCC GT-3’</td>
</tr>
<tr>
<td><strong>RP</strong></td>
<td>5'-CAC GGC CAC GGC CAC GAA CCT GT-3’</td>
<td>5'-CCT AGA AGC ATTT CGG TGC ACG ATG-3’</td>
</tr>
<tr>
<td>Amplicon size:</td>
<td>168</td>
<td>278</td>
</tr>
</tbody>
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4.3.4 **Western blot:**

Western blots were prepared as described in materials and methods. Briefly, both BEAS-2B and A549 cells were grown in T-25 flasks, washed with 1X PBS, harvested and lysed using RIPA buffer [150mM Nacl, 50mM Tris pH 7.5, 1mM EDTA pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 1mM PMSF, 1X Protease inhibitor cock tail]. Samples were incubated on ice 30 min with vortexing at every 10 min during incubation. The samples were then centrifuged at 14,000 rpm for 5 min at 4°C to obtain a clear cell lysate. Total cell lysate protein concentration of both samples was estimated by BCA assay kit as per manufacturer’s instructions. Western blot analysis of total cell lysate with DRD4 specific antibody were carried out as described in chapter 2.
4.3.5 Immunofluorescence assay:
Immunofluorescence analysis of DRD4 in both BEAS-2B and A549 cells were carried out as described earlier in chapter 2.

4.3.6 cAMP assay:
This was done as described previously in chapter 2. Adenylyl cyclase activity was estimated by evaluating the level of the cytosolic cAMP using homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay (LANCE® Ultra cAMP Detection Kit from PerkinElmer Inc) as per manufacturer’s protocol. Reagent preparation & cAMP assay were carried out the same way as described in materials and methods section. In particular cells were incubated with or without quinpirole / PD168077 (DRD4 specific agonist (100nM) in presence and absence of forskolin (10µM) for 30 mins. Cell were also incubated with 1µM spiperone (D2 –like receptor antagonist) in presence of 10µM forskolin and 100nM PD168077. Statistical significance analysis was done by two-tailed, unpaired Student’s t-test using Graphpad prism software version 6.00 (San Diego, CA, USA).

4.4 Results and Discussion:

4.4.1 Expression analysis of DRD4 in human lung cell lines

a) RT-PCR analysis:
Presence of DRD4 mRNA was detected in the total RNA of BEAS-2B and A549 cell lines using gene specific primers (Table 4.1). β-actin was used as an internal control (Fig 4.1).

b) Immunoblot analysis:
Total cell lysates of both BEAS-2B and A549 were subjected to immunoblot analysis using DRD4 specific antibodies. This analysis confirmed the expression of DRD4 in both the cell lines with an immunoreactive band being observed at ~48kDa. No expression was observed in the CHO-K1 cell line, which was used as a negative control (Fig 4.2.).
4.4.2 Localization of DRD4 in human lung cell lines

To determine the localization of DRD4 in plasma membrane of BEAS-2B and A549 cell lines, immunofluorescence analysis was carried out using DRD4 specific antibodies which targets the third extracellular loop of this receptor as described in chapter 2. Localization of this receptor at the cell membrane was observed in both the lung cell lines (Fig 4.3).

4.4.3 Inhibition of adenylyl cyclase activity by DRD4 in human lung cell line:

TR-FRET based estimations of intracellular cAMP concentrations were made following treatment of BEAS-2B cell line with quinpirole/ PD168077 molecules and with forskolin as an inducer. Intracellular level of cAMP which were amplified upon forskolin stimulation were downregulated on an average of ~60% by quinpirole and ~30% by PD68077 in BEAS-2B cells (Fig 4.4) which suggests that DRD4 is expressed in lung tissue and sensitizes the adenylyl cyclase activity up on ligand binding. Further, the DRD4 expression in this lung cell line was confirmed when spiperone (DRD2 specific antagonist) treatment along with PD168077 rescued the activity of adenylyl cyclase from DRD4 mediated inhibition (Fig 4.4). DRD4 is one of main target of antipsychotic drugs, its expression and functional role in brain functions are well discussed and being analysed for its role in neurological and psychiatric disorders being investigated. Various studies have shown the expression of DRD4 receptor in the peripheral system (Ricci et al., 1998 and 2002; Santambrogio et al., 1993; Bondy et al., 1996, Zeng et al., 2008, Zarei et al., 2006, Kitten et al., 2008, Cavallotti et al., 2010). Dopamine receptor types D1 and D2 receptors are reported to be expressed functionally in lung epithelial cells. In this study, we identified and demonstrated the DRD4 mRNA and protein expression for the first time. DRD4 is localized and functionally active (as evidence by the inhibition of adenylyl cyclase activity) at the plasma membrane of human epithelial lung cell lines. Transport of fluid and electrolytes across the epithelium of lung epithelial cells occurs through special polarised transporters at basolateral domain of lung epithelial cells. Transporters like Na\(^+\)K\(^-\)-ATPase are the main gateways for active sodium transport.
across the lung epithelium. Based on the physiological demands, these special transporters including Na\textsuperscript{+}K\textsuperscript{-}-ATPase are activated and regulated by various natural ligands upon their binding to targets. Activation of DRD1 by dopamine has been shown to result in recruitment of intracellular Na\textsuperscript{+}K\textsuperscript{-}-ATPase molecules to plasma membrane thereby increasing this transporter’s activity (Ridge et al., 2002), whereas the activation of D2 receptor increases the transcription rate of Na\textsuperscript{+}K\textsuperscript{-}-ATPase-β1 isoform, which also resulted in increased activity of Na\textsuperscript{+}K\textsuperscript{-}-ATPase (Guerrero et al., 2001). This transcription rate elevation was a result of D2 receptor mediated ERK1/2 phosphorylation and subsequent transcription factor activation (Guerrero et al., 2002).

Further, DRD2 is shown to express and mediate the relaxation of airway smooth muscles (Mizuta et al., 2012). With the above observations of dopamine receptor role in lung epithelial cell electrolyte transport mechanism, and role of clozapine (which has higher affinity towards DRD4 than other dopamine receptor) in peripheral systems (for example, in regulation of insulin secretion (Melkersson et al., 2007), in weight gain (Tschoner et al., 2009), in thrombotic events (Hagg et al., 2002), and their influence in immune function (Agranulocytosis) (Tiihonen et al., 2006)), DRD4 could play an important role in lung epithelial cells as well as in the physiological functions of the peripheral system. However, the mode of action and regulation are yet be explored. Administration of dopamine or of dopamine receptor agonists in both in vivo and in vitro induce vasodilatation in the cerebral, coronary, renal and mesenteric vascular beds and cause hypotension (Amenta et al., 2002) which indicates the necessity of dopamine and its receptor participation. Most of the findings in the peripheral dopamine receptor mechanisms are biased with understanding the role of DRD1 and DRD2 receptors and this is not with case of the other subtypes (DRD3, DRD4, and DRD5). With the proven fact of adverse effects of psychotic drug in the peripheral system, it is necessary to analyse the role of DRD4 in the peripheral system extensively, which could help us to investigate or understand its role in health and disease and for better treatment.

In summary, we demonstrated for the first time the molecular expression of DRD4 in human lung epithelial cell lines and we also demonstrated its functional activity with selective DRD4 agonist mediated adenylyl cyclase inhibition.
**Fig 4.1** Representative gel image of RT-PCR analysis

![Fig 4.1 RT-PCR analysis gel image](Image)

- $\beta$-actin; 278 bp
- DRD4; 168 bp

**Fig 4.2** Expression of DRD4 in human epithelial lung cell lines

![Fig 4.2 DRD4 expression blot](Image)

# The two bands seen in this blot may represent the post translational modifications but this remain to be confirmed
Fig 4.3 Localization of DRD4 in human epithelial lung cell lines

![Image of DRD4 localization in AS49 and BEAS-2B cells]

Fig 4.4 DRD4 mediated inhibition of adenylyl cyclase activity in BEAS-2B cells

![Bar graph showing normalized cAMP levels in response to different treatments]

Data represent Mean ± SD of duplicate points from three independent measurements. ** P < 0.01